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BIOLOGICAL BULLETIN

PUBLISHED BY
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VOLUME 115

AUGUST TO DECEMBER, 1958

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Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P.O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

LANCASTER PRESS, INC., LANCASTER, PA.

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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL] HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk:

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the seventieth session of the Marine Biological Laboratory.

During the past year the Laboratory made significant progress in rehabilitating some of its research space and facilities and also funds were obtained for a new research building and additional housing.

1. *Crane Building*

The Officers of the Laboratory held several conferences during the winter (1957) with representatives of our architectural firm, Coolidge, Shepley, Richardson and Abbott, to develop plans for the rehabilitation of the Crane Building under the National Science Foundation Grant of $415,000. Planning was completed early in the summer; the contracts and subcontracts were let in August. A detailed schedule of the operation was developed by the general contractor, the building was evacuated by the Laboratory immediately after Labor Day and the remodeling started. The schedule called for the completion of the job by May 1, 1958. Present indications are that the work will be completed on time, and that the equipment can be moved in for occupancy by the investigators not later than June 1.

Out of this remodeling the Laboratory will have an essentially new building with facilities for any type of research in biology and the cognate sciences. The rearrangement of the standard facilities within the individual laboratories will result in a much more efficient use of available space.

2. *New Research Laboratory*

In 1938 it was recommended by an *ad hoc* committee set up to formulate policy on the future development of the Marine Biological Laboratory that the wooden buildings should be replaced by a modern brick laboratory building. At the Annual Meeting of the Board of Trustees, August 16, 1957, the Officers of the Laboratory were authorized to seek funds to implement this recommendation. Applications were made to the Rockefeller Foundation, the National Science Foundation and the Public Health Service for the necessary funds. Early in December the Laboratory was notified of a grant from the Rockefeller Foundation of $738,500, providing one-half the necessary funds. Subsequently, grants were obtained from the National Science Foundation and the Public Health Service which shared equally in providing the other half of the cost of the new building and its equipment. Planning for the new building is already under way, construction to start in the fall of 1959 with occupancy planned for the spring of 1961. It will be a three-story and basement building, almost entirely devoted to research and research service laboratories.

3. *Housing*

The 1938 *ad hoc* Committee on the Development of the Laboratory also expressed its concern with the problems of housing and adequate care of the large number of persons attracted to the community by reason of their Laboratory activities. It was pointed out that the housing needs have, from the beginning, been recognized as one of the primary responsibilities of the Laboratory and that the arrangements then existing were not adequate. Since that report, three frame houses in the immediate vicinity of the Laboratory have been acquired and converted to dormitory use. Since World War II there has developed an acute shortage of housing for younger married investigators with children. Toward the end of the year (1957) the Laboratory made application to the National Science Foundation for a grant of $175,000 to erect 25 housekeeping cottages on the Laboratory's Devils Lane Property. Favorable action was taken by the National Science Foundation on this grant request. Plans have been developed for these cottages which will be erected for 1959 occupancy.

Also, the Board of Trustees voted to discontinue any further sale of lots from the Devils Lane Property so that the Laboratory will retain title to the remaining 75 acres for future Laboratory use, either housing or scientific.

4. *Grants, Contracts and Contributions*

The total income to the Laboratory from these sources of support amounted to $210,000 in 1957. This represents 32% of the total income and consists of the following accounts:

American Cancer Soc.—R-7G—Fundamental Studies in Radiobiology	$ 6,600.00
A.E.C.—1343—Program of Research on the Physiology of Marine Organisms Using Radioisotopes	9,545.00
N.I.H.—4359—Biological Research on the Morphology, Ecology, Physiology, Biochemistry and Biophysics of Marine Organisms	40,000.00
N.I.H.—5143—Training Program in Nerve-Muscle Physiology	40,342.00
National Science Found.—G2142—Funds for Biological Research	25,000.00
National Science Found.—G3608—Optical Equipment	11,500.00
National Science Found.—G3987—Centrifuge Equipment	10,000.00
O.N.R.—1497—Studies in Marine Biology	15,000.00
O.N.R.—09701—Studies on Isolated Nerve Fibers	7,670.00
O.N.R.—09702—Studies in Ecology	5,268.00
M.B.L. Associates	3,481.00
Abbott Laboratories	1,000.00
American Philosophical Society	2,500.00
Ciba Pharmaceutical Products, Inc.	1,000.00
Eli Lilly Company	5,000.00
Merck and Company, Inc.	1,000.00
Rockefeller Foundation	20,000.00
Schering Corporation	1,000.00
Smith, Kline, and French Foundation	3,000.00
The Upjohn Company	1,000.00
	$209,906.00

5. *Boats*

Late in the year the Laboratory contracted with the Riverside Boat Company of Newcastle, Maine, for two 24-foot boats for trap work and inshore collecting. These boats will replace the old *Sagitta* and *Tern,* both of which, after years of service, outlived their usefulness. The new boats are to be delivered in May (1958).

Respectfully submitted,
PHILIP B. ARMSTRONG,
Director

MEMORIAL

BENJAMIN M. DUGGAR

by

Wm. Randolph Taylor

Benjamin Minge Duggar, late Emeritus Trustee of this Laboratory, died 10 Sept. 1956 in New Haven, Conn. Dr. Duggar was born in Gallion, Alabama in 1872. His early education in private schools was followed by studies in civil engineering at the University of Alabama and the Mississippi Agricultural and Mechanical College, where his interest shifted to botany, and he received his B.S. in 1891. He then completed his work for the M.S. at Alabama Polytechnic, but, going to Harvard University, qualified there for the A.B. and A.M. degrees, transferring to Cornell University where he received the doctorate in 1898, completing his formal studies. He subsequently worked in several laboratories in Europe, further widening his experience. His government and academic appointments were numerous, but four institutions claimed his services as full Professor before retirement: first the University of Missouri, then Cornell, and then for much longer periods Washington University and the University of Wisconsin.

His distinguished academic career was marked by a happy combination of physiology and pathology, in both of which fields he contributed notably in research and produced textbooks of exceptional merit, that in plant pathology remaining useful to this day. He contributed his share, also, to one of the most successful American elementary botanical text-books ever produced, that prepared by the Wisconsin group and still in use. During his period at Wisconsin the Department of Botany strengthened its position as one of the most notable in the country. His researches covered a considerable range of endeavor, but those on virus diseases, particularly the mosaic disease of tobacco, are most often remembered.

Dr. Duggar does not seem first to have appeared at the Marine Biological Laboratory as a student or as an investigator, as is commonly the case. In 1909 he was appointed to what was termed the "Research Staff" in botany, while Professor of Physiology at Cornell. In 1911 the course in botany was divided, the first three weeks dealing with the algae, the second with "The Physiology and Ecology of Marine, Strand and Bog Vegetation" with Lewis Knudsen, also from Cornell, as his associate. Knudsen was replaced in 1912 by W. J. Robbins, best known as the Director of the New York Botanical Garden, but the course was dropped in 1915. "Investigation Staff" replaced the old term for the advisory group, and Dr. Duggar served Botany on this board from 1926 to 1941. He was elected to the Corporation in 1911 and to the Trustees of the Laboratory in 1928, retiring Emeritus in 1944. During all these years he was frequently in residence through the summer, and always helpful to those at the Laboratory whose enquiries fell within his field of interest.

The discernment shown by Dr. Duggar respecting the affairs of the Marine Biological Laboratory was appreciated by other institutions, and he served as Trustee of the Bermuda Biological Station 1933–1937, and of the Woods Hole Oceanographic Institute from its inception in 1931 until 1938. Honorary degrees were bestowed on him by Washington University, the University of Missouri and the University of Wisconsin; he was elected to the most distinguished of our learned and professional societies.

On retirement from Wisconsin Dr. Duggar promptly joined the research staff of the Lederle Laboratories of the American Cyanamid Company, and settled down to research on the discovery, production and evaluation of antibiotics from various Actinomycete bacteria. All reports from the company describe with admiration his quiet industry and the keen mind he placed most generously at the disposal of his fellow workers. His most spectacular success was in the discovery in 1945 of Aureomycin, a very effective antibiotic, which has gone into extensive commercial production. He continued active in research until his final illness.

Dr. Duggar lost his first wife in 1922; his second wife, several children and grandchildren survive him. To them we wish to express our appreciation of his many contributions to science and our sympathy in the loss they have suffered.

Mr. President, I move that a copy of this memorial be placed in the minutes of this meeting, and that a copy be sent to Mrs. Duggar.

MEMORIAL

E. S. G. Barron

by

H. B. Steinbach

E. S. G. Barron, "Achito" to many, died this summer and is buried in the cemetery at his beloved Woods Hole. His scientific studies achieved world-wide recognition as did the charming personality of the man responsible for them.

While Barron was truly a scientist of the world, his ties to the Marine Biological Laboratory were strong and his affection for the area was great. He was elected a member of the Corporation in 1933, a trustee in 1949 and again in 1952. He served as instructor in the Physiology Course from 1945 until 1948 when he assumed the headship for a five-year period. Under his guidance the course continued its strong development and became especially well known on the international scene. He was largely instrumental in obtaining much of the special equipment that is now in use. He conducted the special session of the course in honor of his revered teacher, Leonor Michaelis, and edited the volume "Modern Trends in Physiology and Biochemistry" which carried the fame of the MBL even farther than before.

For the past several years Barron found it necessary to give up his attendance here to carry out a labor of love dear to his heart, spending his summers in teaching and consulting in South America as his contribution to the advancement of science in those areas, especially the country of his birth, Peru.

While he was perhaps best known for his studies on oxidative mechanisms, Achito's interests and activities were very broad indeed, ranging from a classical work on bilirubinemia to the effects of ionizing radiations on crystalline proteins. However he was preëminently a biologist and, in his mind, all his studies were fundamentally directed at understanding cellular oxidations and their regulation. Shortly before his death, his plans for future work were keyed largely to a comprehensive comparative study of cellular oxidations with the hope of finding critical keys to physiological regulations.

Barron was born in Huari, Peru, in 1898. Following two years in France he came to this country in 1927, first as a Rockefeller Fellow and then as instructor in Johns Hopkins University. In 1930 he moved to the University of Chicago, his University until his death. During World War II, he did scientific work for both the AEC and the Medical Division of the Chemical Warfare Service. He was especially well fitted to carry out the important studies on effects of ionizing radiations and the biological actions of nitrogen mustards and related compounds.

Achito was a remarkable teacher even though his position at Chicago did not involve conducting formal classes. He had a keen and incisive sense of humor and a fine critical attitude towards science. Many have benefitted from his wisdom and have been delighted with his conversation. He had a strong sense of the necessity for training minds in intellectual pursuits. This led him to his fruitful efforts in the Woods Hole course and in the training programs in South America. When he purchased his new home in Chicago some years ago, his greatest delight was that he had a large pleasant room with a big blackboard. Here he could invite his students and colleagues for seminars and discussions and here many of the ideas for experiments by himself and collaborators were born.

Achito, his wife Cora, and his son Richard constituted a family it was a pleasure and privilege to know. And while we are expressing our gratitude to Achito for his many contributions to us, we must include his wife and son for providing the setting for such a fruitful career.

As an experimentalist, as a teacher, as one who travelled widely and spread the traditions of science and inspired the young, Barron was at the height of his activity when he became ill and died. At such a time it is not trite to say that a man's death is untimely.

MEMORIAL

Robert Chambers

by

B. W. Zweifach and G. H. A. Clowes

With the death of Robert Chambers at the age of 75 on July 22nd, the Marine Biological Laboratory lost one of the most illustrious members of its old guard—marking as it were the passing of an era in which microscopy as a fine art was utilized to its fullest extent for the study of cellular behavior and protoplasmic structure. Chambers' associations here in Woods Hole were long and deep-rooted. He first came to the M. B. L. in the summer of 1911 as a graduate investigator and in 1912 was on the teaching staff in Zoology and Embryology—a course in those days associated with such names as Calkins, Lillie, Conklin, Morgan and Wilson. By 1914 it became apparent that Chambers' interests were not along the lines of conventional zoology and he was thereafter listed in the annual reports of the M. B. L. as an investigator in Physiology—an indication that the science of cellular physiology had come of age.

By training Robert Chambers was a histologist and embryologist. He was born and raised in Turkey, where his parents resided as missionaries. The rough, harsh life during his formative years left an indelible imprint on his makeup and was to a considerable extent responsible for his great compassion for the underdog and his willingness to champion humanitarian causes. It was at Roberts College that his interest in nature was crystallized and his future course indicated. Later, under the aegis of Hertwig and Goldschmidt in Munich, where he received his Ph.D in 1908, Chambers was indoctrinated into the field of histophysiology and developed a keen interest in basic embryology. He returned to Canada, the early home of the Chambers family, and eventually joined Cornell Medical College in 1915. These were his most fruitful years—his outstanding contributions in large part derived from his ingenious researches at Woods Hole. His laboratory here in Room 328, in association with the Eli Lilly group, was a beehive of activity where Chambers' dynamic personality infused all who worked with him. Few could keep pace with his amazing physical stamina and drive. At an early age Robert Chambers became virtually a legendary figure, not only because of his scientific stature but because of the anecdotes which grew up around his prodigious unconcern for practical matters. There are many here who knew him during these inspiring years as a most attractive and congenial personality. Woods Hole was the center of the social and scientific life of Robert and Bertha Chambers. They practically raised their four sons at Bobtuckett Cottage and many of the delightful and entertaining experiences of the Chambers family have attained the stature of local folklore. Robert might be found at almost any time, day or night, in his M. B. L. laboratory and the Chambers family regarded the remainder of the year as an unavoidable intrusion into the Woods Hole continuum.

In 1928 he transferred from the Anatomy Department at Cornell to the Department of Biology at New York University, where he maintained until his retirement in 1949 a research center which attracted students from every country of Europe, from Asia, and from South America, many of whom are today outstanding figures in scientific research.

The magnitude of Robert Chambers' contribution becomes all the more impressive when it is considered that he published over 230 scientific articles, bearing in mind the fact that writing was extremely burdensome for Chambers. A great deal of what he did, he left for others to put into words. He unflaggingly, to the point of self-denial, gave his time and counsel to a never-ending stream of students, associates, cronies and visitors. Time was a meaningless entity to him.

In 1912, at the M. B. L. seminar sessions, Chambers was greatly stimulated by a lecture in which G. L. Kite showed that it was possible to interfere with the development of marine ova with glass microtools. In retrospect, we can see that this event proved to be the turning point in his scientific career. The potentialities of this approach appealed so much to Chambers that he developed and applied the microsurgical technique extensively, his name becoming synonymous with the micromanipulative method. In his early work, principally at Woods Hole, he clearly showed the importance of sol-gel transformations in relation to aster formation and cell division. There followed the beautiful demonstrations, accompanied by motion pictures, of the capacity of the cytoplasm and cell surface to recover from various forms of microinjury in the presence of the proper ionic environment. He made the earliest measurements of the pH of the cytoplasm in intact cells, using indicator dyes. His enthusiasm was such that every aspect of cellular behavior intrigued him, the cohesion of blastomeres in developing embryos, the action of salts on protoplasm, the nature of vital staining, the interfacial tension at protoplasmic surfaces, the acid of injury, etc.

Later, Chambers combined tissue culture with microtechniques. Especially noteworthy were his studies on malignant cells, the secretory activity of kidney tubules and chemotactic phenomena. During World War II, he devoted a goodly part of his energies to studies on capillary permeability and to the vascular sequelae of experimental shock. New and important concepts of circulatory homeostasis were originated.

Numerous honors were bestowed upon Chambers. As early as 1926 he gave his first Harvey lecture on the living cell. During this period he received the Traill medal from the Linnean Society of London, the John Scott medal from the City of Philadelphia, the medal of L'Academie Nationale de Médecine of Paris, was made a Fellow of the Royal Microscopical Society of England, and was given an honorary LL.D. from Queens University. He was active in the affairs of many societies, having been a Trustee of the Marine Biological Laboratory, a member of the Board of Directors of the Long Island Biological Association, President of the American Society of Zoologists, the Harvey Society, the Union of American Biological Sciences, and vice-president of the American Association of Anatomists.

When one attempts to give an account of a man's life in a few hundred words, the impossibility of the task becomes increasingly apparent. In the case of Robert Chambers, his human qualities transcended even his outstanding scientific achievements. His later years were saddened by the loss of his oldest son, Robert, in World War II, and by the protracted illness and death of his wife Bertha. It would be a mere platitude to say that we shall miss him, but we hope that the imprint of his indomitable spirit will live on in those of us who were fortunate enough to know him and to contribute some small part to the fruits of his labor.

1. The Staff, 1957

Philip B. Armstrong, Director, State University of New York, School of Medicine, Syracuse

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
A. C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Theodore H. Bullock, Professor of Zoology, University of California, Los Angeles; in charge of course
John M. Anderson, Associate Professor of Zoology, Cornell University
John B. Buck, Senior Biologist, National Institutes of Health
Clark P. Read, Associate Professor, School of Hygiene and Public Health, Johns Hopkins University
Grover C. Stephens, Assistant Professor of Zoology, University of Minnesota
Morris Rockstein, Associate Professor of Physiology, New York University College of Medicine
Cadet Hand, Assistant Professor of Zoology, University of California, Berkeley
Howard A. Schneiderman, Assistant Professor of Zoology, Cornell University

III. Laboratory Assistants

Robert V. Kirchen, Columbia University
Peter Pickens, University of California

EMBRYOLOGY

I. Instructors

M. V. Edds, Jr., Professor of Biology, Brown University; in charge of course
N. T. Spratt, Jr., Professor of Zoology, University of Minnesota
M. Sussman, Associate Professor of Biological Sciences, Northwestern University
J. P. Trinkaus, Associate Professor of Zoology, Yale University
P. B. Weisz, Associate Professor of Zoology, Brown University
E. Zwilling, Program Director, National Science Foundation (on leave from University of Connecticut)

II. Laboratory Assistants

R. G. Beard, Carnegie Institution of Washington, Department of Embryology
C. M. Fulton, Rockefeller Institute for Medical Research

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Györgyi, Director, Institute for Muscle Research, Woods Hole

II. Instructors

W. D. McElroy, Professor of Biology, Johns Hopkins University; in charge of course
Francis D. Carlson, Assistant Professor of Biophysics, Johns Hopkins University
Bernard D. Davis, Professor of Pharmacology, New York University, College of Medicine
Donald Griffin, Professor of Zoology, Harvard University
Howard Schachman, Virus Laboratory, University of California, Berkeley
Andrew Szent-Györgyi, Institute for Muscle Research, Marine Biological Laboratory

III. Laboratory Assistant

Roger Theis, Rockefeller Institute

BOTANY

I. Consultant

Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Harold C. Bold, Professor of Biology, Vanderbilt University; in charge of course
Robert W. Krauss, Associate Professor of Botany, University of Maryland
Richard C. Starr, Associate Professor of Botany, Indiana University

III. Lecturer

Ruth Patrick, Curator of Limnology, Academy of Natural Sciences of Philadelphia

IV. Collector

Gina Arce, Vanderbilt University

V. Laboratory Assistants

Eugene Fox, Indiana University
Raymond A. Galloway, University of Maryland

MARINE ECOLOGY

I. Consultants

Paul Galtsoff, U. S. Fish and Wildlife Service, Woods Hole
Alfred C. Redfield, Woods Hole Oceanographic Institution
John S. Rankin, University of Connecticut

II. Instructors

Eugene P. Odum, Professor of Zoology, University of Georgia; in charge of course
Edwin T. Moul, Associate Professor of Botany, Rutgers University
John H. Ryther, Marine Biologist, Woods Hole Oceanographic Institution

III. Laboratory Assistant

Joanne Van Dyk, University of New Hampshire

THE LABORATORY STAFF, 1957

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
Carl O. Schweidenbach, Manager, Supply Department
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

GENERAL OFFICE

Irvine L. Broadbent, Office Manager

Mrs. Lila S. Myers
Nancy Wigley
Georgiana Marks

LIBRARY

Mary A. Rohan
Naomi Botelho
Albert K. Neal

MAINTENANCE OF BUILDINGS AND GROUNDS

Robert Adams
Edmond A. Botelho
Arthur D. Callahan
Robert Gunning
John H. Head
Donald B. Lehy
Ralph H. Lewis
Russell F. Lewis
Alton J. Pierce
James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

Gail M. Cavanaugh
John P. Harlow
Seaver R. Harlow
Ludie A. Johnson

SUPPLY DEPARTMENT

Donald P. Burnham
Milton B. Gray
Geraldine E. Keeler
Robert M. Perry
Geoffrey J. Lehy
Robert O. Lehy
Bruno Trapasso
H. S. Wagstaff

2. INVESTIGATORS, LALOR AND LILLIE FELLOWS, AND STUDENTS

Independent Investigators, 1957

AIELLO, EDWARD, Assistant in Zoology, Columbia University
ALLEN, M. JEAN, Associate Professor of Biology, Wilson College
ANDERSON, JOHN MAXWELL, Associate Professor of Zoology, Cornell University
ARMSTRONG, PHILIP B., Professor of Anatomy, State University of New York, College of Medicine, at Syracuse
ARNOLD, WILLIAM A., Scientific Investigator, Oak Ridge National Laboratory
BACON, DONALD F., Assistant in Department of Microbiology, Yale Medical School
BANG, FREDERIK, Professor of Pathology, Johns Hopkins University School of Hygiene
BARTON, JAY, II, Associate Professor of Biology, Collegeville, Indiana
BENESCH, REINHOLD, Investigator, Marine Biological Laboratory
BENNETT, MICHAEL, Research Worker, Columbia University, College of Physicians and Surgeons
BENNETT, MIRIAM F., Instructor in Biology, Sweet Briar College
BERG, WILLIAM E., Associate Professor of Zoology, University of California
BERGER, CHARLES A., Chairman, Department of Biology, Fordham University
BISHOP, NORMAN I., Research Associate, University of Chicago
BRADY, ROSCOE, Section Chief, National Institutes of Health
BRAAMS, RENIER, Research Associate, Yale University
BRIDGMAN, JOSEPHINE, Professor of Biology, Agnes Scott College
BROWN, FRANK A., JR., Professor of Biology, Northwestern University
BRYANT, S. H., Professor of Pharmacology, University of Cincinnati, College of Medicine
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CARLSON, FRANCIS D., Associate Professor of Biophysics, Johns Hopkins University
CASE, JAMES F., Assistant Professor of Zoology, Iowa State University
CHAET, ALFRED B., Instructor in Physiology, Boston University School of Medicine
CHANG, JOSEPH J., Member of Laboratory of Biophysics, National Institutes of Health
CHASE, AURIN M., Associate Professor of Biology, Princeton University
CHENEY, RALPH H., Professor of Biology; Director Physiology Division, Brooklyn College
CLAFF, C. LLOYD, Research Associate in Surgery, Harvard Medical School
CLARK, GORDON M., Research Associate, University of Michigan
CLEMENT, A. C., Professor of Biology, Emory University
CLOWES, G. H. A., Research Director Emeritus, Lilly Research Laboratories
COLE, KENNETH S., Chief, Laboratory of Biophysics, National Institutes of Health
COHEN, MELVIN J., Instructor in Biology, Harvard University
COLLIER, JACK R., Instructor in Zoology, University of Vermont
COLWIN, ARTHUR L., Associate Professor, Queens College
COLWIN, LAURA HUNTER, Lecturer, Queens College
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CROWELL, SEARS, Associate Professor of Zoology, Indiana University
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ELLIOTT, ALFRED M., Professor of Zoology, University of Michigan

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Failla, G., Professor, Columbia University
Frye, B. E., Postdoctoral Fellow, Princeton University
Gall, Joseph G., Assistant Professor of Zoology, University of Minnesota
Green, Howard, Assistant Professor of Chemical Pathology, New York University College of Medicine
Green, Maurice, Assistant Professor of Microbiology, St. Louis University School of Medicine
Greenberg, Sylvia S., Damon Runyon Cancer Research Fellow, New York University
Gregg, James H., Associate Professor of Biology, University of Florida
Greif, Roger L., Associate Professor of Physiology, Cornell University Medical College
Grosch, Daniel S., Associate Professor of Genetics, N. C. State College
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Harvey, Ethel Browne, Research in Biology, Princeton University
Harvey, E. Newton, Professor Emeritus of Biology, Princeton University
Hayashi, Teru, Associate Professor of Zoology, Columbia University
Heilbrunn, L. V., Professor of Zoology, University of Pennsylvania
Henley, Catherine, Research Associate, University of North Carolina
Hervey, John P., Electronic Engineer, Rockefeller Institute for Medical Research
Hill, Robert B., Instructor in Zoology, University of Maine
Hoberman, Henry D., Associate Professor of Biochemistry, Albert Einstein College of Medicine
Horowitz, Samuel B., Research Fellow, Eastern Pennsylvania Psychiatric Institute
Hoyle, Graham, Glasgow University, Scotland
Hyde, Beal B., Assistant Professor of Plant Sciences, University of Oklahoma
Isenberg, Irvin, Research Associate, Institute for Muscle Research
Jenner, Charles E., Associate Professor of Zoology, University of North Carolina
Kalckar, Barbara W., Biochemist, National Institutes of Health
Kemp, Norman E., Assistant Professor of Zoology, University of Michigan
Kempton, Rudolf T., Professor of Zoology, Vassar College
Klotz, Irving M., Professor of Chemistry and Biology, Northwestern University
Kuffler, Stephen W., Professor of Ophthalmic Physiology and Biophysics, Johns Hopkins University
Lansing, Albert I., Professor of Anatomy, University of Pittsburgh
Lazarow, Arnold, Professor and Head of Department of Anatomy, University of Minnesota
Lawler, H. Clair, Associate in Biochemistry, College of Physicians and Surgeons
Lawrence, H. Sherwood, Associate Professor of Medicine, New York University College of Medicine
Leighton, Joseph, Assistant Professor of Pathology, University of Pittsburgh
Levy, Milton, Professor and Chairman, Department of Biochemistry, New York University College of Dentistry
Lewin, Ralph A., National Institutes of Health
Lindberg, Olov, Professor and Head of Wenner-Grens Institute, Sweden
Litt, Mortimer, Research Fellow in Bacteriology, Harvard Medical School
Lochhead, John H., Professor of Zoology, University of Vermont
Lorand, L., Assistant Professor of Chemistry, Northwestern University
Lowenstein, O. E., Professor of Zoology, University of Birmingham, England
Lubin, Martin, Associate in Pharmacology, Harvard Medical School
McElroy, W. D., Chairman, Biology Department, Johns Hopkins University
Maas, Werner K., Assistant Professor of Pharmacology, New York University Medical College
Marshak, Alfred, Marine Biological Laboratory
Marsland, Douglas, Professor of Biology, New York University, Washington Square College
Menkin, Valy, Head of Experimental Pathology, Temple University School of Medicine
Metz, Charles B., Associate Professor of Zoology, Florida State University
Metz, Charles W., Professor of Zoology, University of Pennsylvania
Middlebrook, W. Robert, Institute for Muscle Research

Mills, Kenneth S., Instructor of Biophysics, University of California Medical Center
Moore, John W., Associate Chief, National Institutes of Health
Mulnard, Jacques G., Chef De Travau, University of Brussels, Belgium
Mullins, L. J., Associate Professor of Biophysics, Purdue University
Nace, Paul F., Associate Professor of Biology, Hamilton College, McMaster University, Ontario
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Odum, Eugene P., Professor of Zoology, University of Georgia
Osterhout, W. J. V., Member Emeritus, Rockefeller Institute for Medical Research
Padawer, Jacques, Assistant Professor of Biochemistry, Albert Einstein College of Medicine
Parpart, Arthur K., Professor and Chairman, Department of Biology, Princeton University
Person, Philip, Chief, Dental Research, V. A. Hospital, Brooklyn
Pert, James H., Professor in Medicine, Cornell University Medical College
Plough, Harold H., Professor of Biology, Amherst College
Prosser, C. Ladd, Professor of Physiology, University of Illinois
Read, Clark P., Associate Professor of Pathobiology, Johns Hopkins University
Rebhun, Lionel I., Instructor in Anatomy, University of Illinois College of Medicine
Rieser, Peter, Research Associate, University of Pennsylvania
Rockstein, Morris, Associate Professor of Physiology, New York University College of Medicine
Rogers, K. T., Assistant Professor of Zoology, Oberlin College
Rosenberg, Evelyn K., Assistant Professor of Pathology, New York University-Bellevue Medical Center
Roth, Jay S., Associate Professor of Biochemistry, Hahnemann Medical College
Rugh, Roberts, Associate Professor of Radiology, Columbia University
Schechter, Victor, Associate Professor of Biology, City College of New York
Schneiderman, Howard A., Associate Professor of Zoology, Cornell University
Schoffeniels, Ernest, Research Associate, College of Physicians and Surgeons
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Scott, Dwight B. McNair, Assistant Professor of Physiology, University of Pennsylvania Medical School
Scott, Sister Florence Marie, Professor and Chairman, Department of Biology, Seton Hill College
Scott, George T., Professor of Zoology, Oberlin College
Shanes, A. M., Physiologist, National Institutes of Health
Shaw, Evelyn, Research Fellow, American Museum of Natural History
Slifer, Eleanor H., Associate Professor of Zoology, State University of Iowa
Smelser, George K., Professor of Anatomy, College of Physicians and Surgeons
Speidel, Carl C., Professor and Chairman, Department of Anatomy, University of Virginia
Sperelakis, Nick, Teaching Assistant, University of Illinois
Spiegel, Melvin, Assistant Professor of Biology, Colby College
Spratt, Nelson T., Professor of Zoology, University of Minnesota
Spyropoulos, Constantine, National Institutes of Health
Starr, Richard C., Assistant Professor of Botany, Indiana University
Steele, Richard H., Institute for Muscle Research
Steinbach, H. B., Professor of Zoology, University of Chicago
Stephens, Grover C., Assistant Professor of Zoology, University of Minnesota
Stephenson, William K., Assistant Professor of Biology, Earlham College
Stetten, DeWitt, Associate Director in Charge of Research, National Institutes of Health
Stokey, Alma G., Professor Emeritus, Mount Holyoke College
Stone, William, Jr., Massachusetts Eye and Ear Infirmary
Stunkard, Horace W., Research Biologist, U. S. Fish and Wildlife Service
Sussman, Maurice, Associate Professor of Biological Sciences, Northwestern University
Szent-Györgyi, Albert, Chief Investigator, Institute for Muscle Research
Szent-Györgyi, Andrew G., Investigator, Institute for Muscle Research
Tasaki, Tchiji, Chief, Special Senses Section, National Institutes of Health

TAYLOR, WILLIAM RANDOLPH, Professor of Botany, University of Michigan
TEWINKEL, LOIS E., Professor of Zoology, Smith College
TRINKAUS, JOHN PHILIP, Associate Professor of Zoology, Yale University
TROLL, WALTER, Assistant Professor, New York University College of Medicine
TWAROG, BETTY MACK, Research Fellow, Harvard University
TWEEDELL, KENYON S., Assistant Professor of Zoology, University of Maine
ULLBERG, SVEN G. F., Royal Veterinary College, Stockholm, Sweden
DEVILLLAFRANCA, GEORGE W., Assistant Professor of Zoology, Smith College
VILLEE, CLAUDE A., Associate Professor of Biological Chemistry, Harvard Medical School
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WAINIO, WALTER W., Associate Professor of Biochemistry, Rutgers University
WEBB, H. MARGUERITE, Assistant Professor of Physiology, Goucher College
WEIGLE, WILLIAM O., Research Associate, University of Pittsburgh School of Medicine
WESTHEIMER, GERALD, Assistant Professor of Physiological Optics, Ohio State University
WHITING, ANNA R., Lecturer in Zoology, University of Pennsylvania
WHITING, P. W., Professor of Zoology Emeritus, University of Pennsylvania
WICHTERMAN, RALPH, Professor of Biology, Temple University
WILBER, CHARLES G., Chief, Comparative Physiology Branch, Army Chemical Center
WILLEY, C. H., Chairman, Department of Biology, New York University
WILSON, DONALD M., Teaching Assistant, University of California, Los Angeles
WILSON, T. HASTINGS, Assistant Professor of Biological Chemistry, Washington University School of Medicine
WILSON, WALTER L., Assistant Professor of Physiology, University of Vermont College of Medicine
WITTENBERG, JONATHAN B., Assistant Professor of Physiology, Albert Einstein College of Medicine
WOODS, KENNETH R., Research Associate, Cornell University Medical College
WRIGHT, PAUL A., Associate Professor of Zoology, University of Michigan
ZWEIFACH, BENJAMIN W., Associate Professor of Pathology, New York University-Bellevue Medical Center
ZWILLING, EDGAR, Associate Professor of Genetics, University of Connecticut

Beginning Investigators, 1957

ALSUP, PEGGY, University of Pennsylvania
BENSUSAN, HOWARD B., Western Reserve University
BURKE, JOSEPH, S.J., Fordham University
CAGLE, JULIEN, Princeton University
CASCARANO, JOSEPH, University of Minnesota Medical School
CERT, JEAN A., University of California
CHANCE, ELEANOR K., University of Pennsylvania
DINGLE, A. D., McMaster University
GANGI, DOMINICK P., Upstate Medical Center, State University of New York
HARDIMAN, CLARENCE W., Florida State University
KANE, ROBERT E., Johns Hopkins University
MASHIMA, HIDENOBER, Rockefeller Institute for Medical Research
MASON, DAVID T., Reed College
MOOS, CARL, Northwestern University
NAGLER, ARNOLD L., Bellevue Medical Center
ROSS, SAMUEL M., State University of New York, College of Medicine at Brooklyn
RUGGIERI, GEORGE, St. Louis University
SCHWARTZ, JAMES H., New York University College of Medicine
SMITH, ROBERT G., Washington University Medical School
STROHMAN, RICHARD C., Columbia University
THEIS, ROGER E., Rockefeller Institute for Medical Research
TURNER, BARBARA, Johns Hopkins University School of Medicine
WESSELLS, NORMAN KEITH, Yale University

Research Assistants, 1957

Albert, Morris, Boston University
Amatniek, Ernest, Columbia University
Auclair, Walter, New York University, Washington Square College
Barnhart, B. J., Indiana University
Barnwell, Franklin H., Northwestern University
Barrow, Patience C., University of Toronto
Benoit, Richard, Massachusetts Eye and Ear Infirmary
Blanchard, Roberta, Woods Hole, Mass.
Bruck, Stephen D., duPont de Nemours & Company
Cather, James N., Emory University
Clark, William R., Jr., Boston University
Corner, M., Rockefeller Institute
Crowley, Elizabeth M., University of Pittsburgh
Dibbell, David G., University of Pennsylvania
Douglas, Steven, Cornell University
Erdman, Howard E., North Carolina State College
Feinberg, Harriet Adele, University of Pennsylvania
Feldman, Richard, Rockefeller Institute for Medical Research
Fox, J. Eugene, Indiana University
Friedman, Leonard, Rutgers University
Gebhart, John H., National Institutes of Health
Gifford, Cameron E., Harvard University
Gifford, Charles A., University of Minnesota
Gordon, Robert, Massachusetts Institute of Technology
Gorkenant, Ingeburg, Woods Hole, Mass.
Goudsmit, Esther M., University of Michigan
Grinnell, Alan, Harvard University
Hiatt, Howard, Harvard Medical School
Inglis, Laura H., Hahnemann Medical College
Jones, Helen, Massachusetts Eye and Ear Infirmary
Josephson, Robert K., University of California
Karakashian, Stephen J., Drew University
Kaufman, Sharon L., Smith College
Kerevyi, Thomas, Harvard Medical School
Kernan, Roderick P., Rockefeller Institute for Medical Research
Kirchen, Robert V., Columbia University
Kopman, Aaron, Queens College
Krassner, Stuart, Johns Hopkins University
Levi, Colette P., Northwestern University
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Loring, Janet, Harvard Medical School
Luhrs, Caro, Harvard Medical School
Matheson, Gail E., Yale University
McCann, Francis, University of Connecticut
Metcalf, Carroll, Colby College
Morrison, Elaine, Massachusetts Eye and Ear Infirmary
Nass, Sylvan, New York University
Olson, Joanne M., University of Minnesota
Paulsen, Elizabeth, Rutgers University
Plumb, Mary Ellen, Vassar College
Pollock, Brian, Brooklyn V. A. Hospital
Reich, Melvin, Rutgers University
Richards, Elmer G., University of California
Robertson, Mrs. C. W., United States Fish and Wildlife Service
Root, Richard, University of Michigan
Root, Elizabeth, University of Michigan

Rosenbluth, Raja, Columbia University
Ross, Shirley Eileen, Washington University
Rossillo, Ludwig A., St. Peter's College
Rubinoff, Ira, American Museum of Natural History
Schinske, Robert, University of Minnesota
Scheltema, Amelie H., University of North Carolina
Shay, Jonathan, Temple Medical School
Shepard, David, University of Chicago
Simmons, John E., Johns Hopkins University
Smiley, Sheldon, New York State University at Syracuse
Stadler, Joan, Swarthmore College
Staub, Herbert W., Rutgers University
Titus, Charles C., Western Reserve University
Tremor, John, University of Michigan
Waite, Richard E., University of Pennsylvania
Warwick, Anne C., Johns Hopkins University
Weisblum, Bernard, State University of New York
Wellington, Frederica, Harvard Medical School
Whitbeck, Elaine, Smith College
Wyttenbach, Charles R., Carnegie Institute

Library Readers, 1957

Allfrey, Vincent G., Associate, Rockefeller Institute for Medical Research
Amberson, William R., Professor of Physiology, University of Maryland School of Medicine
Ball, Eric G., Chairman, Division of Medical Sciences, Harvard Medical School
Bernheimer, Alan W., Associate Professor of Microbiology, New York University College of Medicine
Bloch, Robert, Associate Editor, *Biological Abstracts,* University of Pennsylvania
Bodansky, Oscar, Sloan-Kettering Institute
Brooks, Matilda M., Research Associate in Physiology, University of California
Chanutin, Alfred, Professor of Biochemistry, Medical School, University of Virginia
Clark, Eliot R., Professor Emeritus of Anatomy, University of Pennsylvania School of Medicine
Cohen, Seymour S., Professor of Biochemistry, Children's Hospital
Deane, Helen Wendler, Harvard Biological Laboratories
Dixon, Frank J., Jr., Chairman, Department of Pathology, University of Pittsburgh School of Medicine
DuBois, Arthur D., Associate Professor of Physiology, University of Pennsylvania School of Medicine
Eichel, Herbert J., Hahnemann Medical College
Eisen, Herman N., Professor of Medicine, Washington University
Gabriel, Mordecai L., Associate Professor of Biology, Brooklyn College
Gaffron, Hans, Professor of Biochemistry, University of Chicago
Goldthwait, David A., Western Reserve University
Green, James W., Associate Professor of Physiology, Rutgers University
Jacobs, M. H., Emeritus Professor of General Physiology, University of Pennsylvania School of Medicine
Kaan, Helen W., Indexer, National Research Council
Karush, Fred, Associate Professor of Immunology, University of Pennsylvania
Lionetti, Fabian J., Associate Professor of Biochemistry, Boston University School of Medicine
London, Irving M., Professor and Chairman, Department of Medicine, Albert Einstein College of Medicine
Love, Lois H., Research Associate, National Research Council
McDonald, Sister Elizabeth, Chairman, Department of Biology, College of Mt. St. Joseph
Moore, George M., Professor and Chairman of Zoology, University of New Hampshire
Novikoff, Alex B., Research Associate Professor of Pathology, Albert Einstein College of Medicine

Pick, Joseph, Professor of Anatomy, New York University-Bellevue Medical Center
Root, Walter S., Professor of Physiology, College of Physicians and Surgeons
Rose, S. Meryl, Professor of Zoology, University of Illinois
Schlesinger, R. Walter, Director, Department of Microbiology, St. Louis University School of Medicine
Scott, Allan, Professor of Biology and Chairman of Department, Colby College
Sherman, Frank E., Assistant Professor of Pathology, University of Pittsburgh
Steinhardt, Jacinto, Director, Operations Evaluation Group, Massachusetts Institute of Technology
Sulkin, S. Edward, Professor and Chairman, Department of Microbiology, University of Texas, Southwestern Medical School
Wagner, Robert R., Assistant Professor of Medicine, Johns Hopkins University School of Medicine
Warner, Robert C., Associate Professor of Biochemistry, New York University College of Medicine
Wheeler, George E., Instructor of Biology, Brooklyn College
Whitehouse, Michael W., Instructor of Biochemistry, University of Pennsylvania School of Medicine
Yntema, Chester L., Professor of Anatomy, State University of New York College of Medicine
Zorzoli, Anita, Associate Professor of Physiology, Vassar College

LALOR FELLOWS, 1957

Bacon, Donald, Yale Medical School
Bishop, Norman, University of Chicago
Bryant, S. H., University of Cincinnati
Bückmann, Detlef, Zoologisches Institut, Mainz, Germany
Burgen, A. S. V., McGill University
Edwards, Charles, Johns Hopkins University
Engle, Ralph, Cornell University Medical College
Lorand, L., Northwestern University
Lindberg, Olov, Wenner-Grens Institute, Sweden
Lubin, Martin, Harvard Medical School
Schulman, Martin, State University of New York, College of Medicine at Syracuse
Stephenson, W. K., Earlham College
Whitehouse, Michael, University of Pennsylvania School of Medicine
Wilson, T. Hastings, Washington University School of Medicine
Woods, Kenneth, Cornell University Medical School

Lillie Fellow, 1957

Niu, Man-Chiang, Rockefeller Institute for Medical Research

Students, 1957

BOTANY

Abeles, Fred B., Cornell University
Arnold, Elizabeth I., University of Rochester
Aronson, Flora P., Brooklyn College
Bouck, George B., Columbia University
Cook, Philip W., University of Vermont
Czelusniak, Marilyn M., Smith College
Frankel, Joseph, Yale University
Herskowitz, Julia, Antioch College
Keeler, Carl R., Jr., Northwestern University
Klepper, Elizabeth, Vanderbilt University

Kuenzler, Edward J., University of Georgia
Moreland, Ralph E., Jr., Indiana University
Muschio, Henry M., Fordham University
Paik, Hyangju, Wellesley College
Paoli, Gisela, Chatham College
Parker, Bruce C., Yale University
Prota, Carl D., Fordham University
Rice, Eleanor, Wheaton College
Tews, Leonard C., Indiana University
Walser, Stephanie L., Radcliffe College

EMBRYOLOGY

Arkless, Richard, University of Pennsylvania Medical School
Caston, J. Douglas, University of North Carolina
Goeringer, Gerald C., Johns Hopkins University
Griffin, Joe Lee, Princeton University
Hanks, James E., University of New Hampshire
Hersh, George L., University of California
Karakashian, Stephen J., Drew University
Kerr, Norman S., Northwestern University
Kessel, Richard G., State University of Iowa
Kirchen, Robert V., Columbia University
Kram, Fleurette L., Northwestern University
Love, David S., University of Colorado
Lowe, Janet M., University of Minnesota
Mathiesen, George C., Harvard University
Mellon, DeForest, Jr., Yale University
Nelson, Shirley, Northwestern University
Rosewater, Joseph, Harvard University
Sparano, Benjamin M., Fordham University
Talbot, William H., Rockefeller Institute
Tyson, Greta E., University of New Hampshire
Van Dyk, N. Joanne, University of New Hampshire
Walcott, Charles, Cornell University
Watkins, Margaret J., University of Minnesota
White, Jean Ann, Mount Holyoke College
Wylie, Richard M., Harvard University

PHYSIOLOGY

Clark, Alvin John, Harvard University
Cox, Rody P., University of Pennsylvania
Davidson, Morton, Bellevue Medical College
Dubnau, David A., Columbia University
Erwin, Joseph A., Syracuse University
Fahn, Stanley, University of California School of Medicine
Felix, Marie D., Cornell University Medical School
Haft, David E., University of Rochester School of Medicine
Halperen, Sidney, University of Texas
Kahlbrock, Margit, Columbia University
Kirsch, Jack F., Rockefeller Institute
McCluskey, Robert T., New York University-Bellevue Medical Center
Mazur, Peter, Princeton University
Medina, Heitor S., Inst. de Biolojia—Curitiba, Paroni, Brazil
Mindich, Leonard E., Rockefeller Institute
Nagler, Arnold L., Bellevue Medical School
Otero, Luis R., University of Puerto Rico
Rabinowitz, Lawrence, University of California
Rawitscher, Erika, American Museum of Natural History

Roberts, Patricia R., Duke University
Schneider, John H., University of Wisconsin
Siger, Alvin, Johns Hopkins University
Stern, Daniel N., Albert Einstein College of Medicine
Stone, Nancy J., Columbia University
Takeuchi, Ikuo, Princeton University
Weeks, Boyd M., University of California
Williams, Frank Robert, Oberlin College
Williams, Frederick M., Yale University
Willis, John S., Harvard University

INVERTEBRATE ZOOLOGY

Asher, David M., Harvard University
Augenfeld, John M., University of Wisconsin
Becker, Joyce E., Evansville College
Branning, Arleen, City College of New York
Braverman, Maxwell H., University of Illinois
Camp, Donald B. M., Acadia University
Clarke, Arthur H., Jr., Cornell University
Coleman, Chase, Vassar College
Concannon, Bro. Joseph, St. John's University
Cooper, Madeline, American Museum of Natural History
Cooper, Kenneth K., American Museum of Natural History
Crowell, Jane, Oberlin College
Diamond, Jared M., Harvard University
Dobben, Phyllis A., Rocky River 16, Ohio
Dobbs, Harry D., Wofford College
Egloff, David A., Amherst College
Gfeller, Sister Marion D., Marquette University
Guze, Carol D., Washington University
Hafener, Paul A., Jr., Franklin and Marshall College
Hechtel, George J., Yale University
Hild, David H., Wesleyan University
Horvath, Nancy, 10121 S. Parnell Avenue, Chicago 38, Illinois
Horwitz, Judith, Radcliffe College
Isaac, Donald E., University of California
Jensen, Donald Dale, Yale University
Johnson, B. Thomas, University of California
Jordan, Elke, Goucher College
Kaufman, John H., University of California
Krassner, Stuart, Johns Hopkins University
Lane, Rosemary M., Dalhousie University
Leisy, Elsa, University of California
Longacre, Harriette, Mount Holyoke College
Lorenzo, Michael A., St. Louis University
Lowe, Mildred E., Tulane University
McManus, Lawrence Robert, Cornell University
Menaker, Michael, Princeton University
Newberry, Andrew Todd, Stanford University
Poulson, Thomas L., University of Michigan
Prager, Jan C., University of Cincinnati
Reese, Ernst S., University of California
Root, Richard B., University of Michigan
Ross, Shirley E., Washington University
Sherman, Irwin W., City College of New York
Smith, S. Clarke, Wabash College
Smith, Susan, Earlham College
Thompson, Jane F., University of Massachusetts
Thompson, Martha Jane, Oberlin College

Trotter, Nancy L., Brown University
Vitols, Andris T., University of Minnesota
Wilhelm, Robert C., Cornell University
Willis, John S., Harvard University
Wittry, Sister Esperance, College of St. Catherine
Wood, Langley H., Cornell University
Yow, Francis W., Emory University

ECOLOGY

Abeles, Fred, Cornell University
Barber, Richard I., Brown University
Barth, Robert H., Jr., Harvard University
Blunt, Sister Marion Xavier, Marquette University
Bothner, Richard C., Fordham University
Ellsworth, Joanne, Elmira College
Gifford, Cameron E., Harvard University
Randall, Donald, Oberlin College
Story, Lawrence P., Drew University

3. Fellowships and Scholarships, 1957

Lucretia Crocker Scholarship:
George B. Bouck, Botany Course
Conklin Scholarship:
Robert Kirchen, Embryology Course
Merkel Jacobs Scholarship:
Margit Kahlbrock, Physiology Course
Calkins Scholarship:
Thomas L. Poulson, Invertebrate Zoology Course
Bio Club Scholarships:
Arleen Branning, Invertebrate Zoology Course
Irwin W. Sherman, Invertebrate Zoology Course
Linton Memorial Fund:
C. D. Dieter, Washington-Jefferson College

4. Tabular View of Attendance, 1953–1957

	1953	*1954*	*1955*	*1956*	*1957*
Investigators—Total	310	298	250	304	326
Independent	176	180	162	184	186
Under Instruction	37	20	9	20	23
Library Readers	46	52	54	50	42
Research Assistants	51	46	25	50	75
Students—Total	136	134	148	140	139
Zoology	55	56	56	55	55
Embryology	30	29	30	28	27
Physiology	31	28	30	30	30
Botany	11	12	19	18	18
Ecology	9	9	13	9	9
Total Attendance	446	432	398	444	465
Less persons represented as both investigators and students		5		2	3
	446	427	398	442	462

Institutions Represented—Total	155	136	129	130	129
By investigators	90	104	95	97	94
By students	65	32	34	33	35
Schools and Academies Represented					
By investigators		2	3	3	5
By students	1	1	2	1	1
Foreign Institutions Represented					
By investigators	15	11	8	9	11
By students	6	13	6	6	5

5. Institutions Represented, 1957

Amherst College
American Museum of Natural History
Boston University School of Medicine
Brooklyn College
Brown University
Bryn Mawr College
Chatham College
Children's Hospital of Philadelphia
City College of New York
Colby College
College of Mt. St. Joseph on the Ohio
Columbia University, College of Physicians and Surgeons
Columbia University, Zoology Dept.
Cornell University
Cornell University Medical School
Corporation of Roman Catholic Clergymen
Duke University
Albert Einstein College of Medicine
Elmira College
Emory University
Florida State University
Fordham University
Hahnemann Medical College
Harvard University
Harvard University Medical School
Indiana University
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly and Company
Marquette University
National Institutes of Health
New York University—Heights
New York University College of Medicine
New York University, Washington Square College
North Carolina State College
Northwestern University
Oberlin College
Princeton University
Purdue University
Radcliffe College
Rockefeller Institute for Medical Research
Rutgers University
Saint Joseph's College
St. Louis University
St. Louis University, School of Medicine
Single Cell Foundation
Sloan-Kettering Institute
Southwestern Medical College
State University of Iowa
State University of New York, College of Medicine at Syracuse
Syracuse University
Temple University
University of Chicago
University of Florida
University of Illinois
University of Illinois, College of Medicine
University of Maine
University of Michigan
University of Minnesota
University of New Hampshire
University of North Carolina
University of Oklahoma
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Vermont
University of Virginia, School of Medicine
University of Wisconsin
U. S. Fish and Wildlife Service
Vassar College
Veterans Administration Hospital of Brooklyn
Wabash College
Washington and Jefferson College
Washington University School of Medicine
Wellesley College
Wesleyan University
Wheaton College
Wilson College
Yale University

Supporting Institutions and Agencies, 1957

Abbott Laboratories
American Cancer Society
American Philosophical Society
Associates of the Marine Biological Laboratory
Atomic Energy Commission
Ciba Pharmaceutical Products, Inc.
The Grass Foundation
Kellogg Foundation
The Lalor Foundation
Eli Lilly and Company
Merck and Company, Inc.
National Institutes of Health
National Science Foundation
Office of Naval Research
The Rockefeller Foundation
Schering Corporation
Smith, Kline and French Foundation
The Upjohn Company

Foreign Institutions Represented, 1957

Zoologisches Institut, Mainz, Germany
McGill University, Montreal, Canada
Glasgow University, Scotland
University College, London, England
Wenner-Grens Institute, Sweden
University of Birmingham, England
University of Oslo, Sweden
University of Brussels, Belgium
Royal Veterinary College, Sweden
Utrecht University, The Netherlands
McMaster University, Hamilton College, Canada

6. Evening Lectures, 1957

July 5	
Bentley Glass	"In pursuit of a gene"
July 12	
K. Linderstrøm-Lang	"Deuterium exchange of proteins in aqueous solution"
July 19	
Olov Lindberg	"Functional-structural correlations in mitochondria"
July 26	
Albert I. Lansing	"Chemical morphology of the elastic fiber"
August 2	
James D. Ebert	"The acquisition of biological specificity"
August 9	
J. C. Eccles	"The behavior of nerve cells"
August 16	
Francis J. Ryan	"Mutation as an error in gene duplication"
August 23	
Seymour S. Cohen	"The chemical pathology of the virus infected cell"

7. Tuesday Evening Seminars, 1957

July 2	
Charles B. Metz	"The enhancement of starfish sperm motility and respiration by metals and metal binding agents"
Norman E. Kemp	"Differentiation of cortical cytoplasm and extra-cellular membranes of oocytes, including changes at fertilization"

Laura Hunter Colwin and Arthur L. Colwin	"Lytic and other activities of the individual spermatozoon during the early events of sperm entry (Hydroides, Saccoglossus, and several other invertebrates)"
July 9	
A. M. Shanes	"Ion movement in vertebrate nerve"
William Stephenson	"Relationships between ion movements and membrane potential changes in muscle"
G. Hoyle	"Nervous control of muscular contraction in arthropods"
W. H. Freygang, Jr.	"Evidence for electrical inexcitability of neuron soma"
July 16	
T. R. Tosteson, S. A. Ferguson and L. V. Heilbrunn	"Further studies of the antimitotic and carcinostatic action of ovarian extracts"
L. V. Heilbrunn, Francis Ashton, Carl Feldherr and W. L. Wilson	"The action of insulin on living cells"
Francis Ashton	"Magnetic studies on cells and protoplasm"
Carl Feldherr	"The metachromatic reaction in various types of protoplasm"
Peter Rieser	"Effect of x-rays on fibrinogen"
Paul R. Gross, Sylvan Nass and William Pearl	"Mechanisms of sol-gel transformations in the cytoplasm"
July 23	
R. E. Benesch and R. Benesch	"Sulfur linkages in hemoglobins"
A. Chase	"Uricase inactivation by urea"
L. Lorand	"Clotting of blood: a study of the polymerization of proteins"
H. K. Schachman	"Structural considerations on bushy stunt virus"
July 30	
Luigi Provasoli	"Effect of plant hormones on sea weed"
Dwight McNair Scott	"Changes in RNA during synchronous division of E. coli"
Jay S. Roth	"Observations on the RNase system of rat liver"
Bernard Davis	"Bacterial permease systems"
August 6	
Joseph Gall	"Thymidine incorporation into the macronucleus of Euplotes (Protozoa)"
Beal B. Hyde	"The effect of Versene on the sulfhydryls of chromatin"
C. W. Metz	"Interactions between chromosomes and cytoplasm during early embryonic development in Sciara (Diptera)"

August 13	
Bostwick H. Ketchum	"Marine ecology and its place in biological research"
Eugene P. Odum	"Studies on simple natural ecosystems"
John H. Ryther	"On the efficiency of photosynthesis in the sea"
Thomas S. Austin	"The ecology of the biota of the equatorial Pacific"
August 20	
L. Lorand, J. Molnar and C. Moos	"Biochemical studies of relaxation in glycerinated muscle"
F. D. Carlson and A. Siger	"Creatine phosphate and adenosintriphosphate breakdown in iodoacetate poisoned muscle"
A. G. Szent-Györgyi and Carolyn Cohen	"Structural aspects of muscle proteins"
T. Hayashi, R. Strohman and R. Rosenbluth	"Myosin and actin interaction, and construction"

8. Members of the Corporation, 1957

1. Life Members

Brodie, Mr. Donald M., 522 Fifth Avenue, New York 18, New York
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania
Carver, Dr. Gail L., Mercer University, Macon, Georgia
Cole, Dr. Elbert C., 2 Chipman Park, Middlebury, Vermont
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri
Crane, Mrs. W. Murray, Woods Hole, Massachusetts
Dederer, Dr. Pauline H., Connecticut College, New London, Connecticut
Dungay, Dr. Neil S., Carleton College, Northfield, Minnesota
Goldfarb, Dr. A. J., College of the City of New York, New York City, New York
Knowlton, Dr. F. P., 1356 Westmoreland Avenue, Syracuse, New York
Lewis, Dr. W. H., Johns Hopkins University, Baltimore, Maryland
Lowther, Dr. Florence deL., Barnard College, New York City, New York
MacNaught, Mr. Frank M., Woods Hole, Massachusetts
Malone, Dr. E. F., 6610 North 11th Street, Philadelphia 26, Pennsylvania
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts
Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pennsylvania
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania
Riggs, Mr. Lawrason, 74 Trinity Place, New York 6, New York
Scott, Dr. Ernest L., Columbia University, New York City, New York
Turner, Dr. C. L., Northwestern University, Evanston, Illinois
Waite, Dr. F. G., 144 Locust Street, Dover, New Hampshire
Wallace, Dr. Louise B., 359 Lytton Avenue, Palo Alto, California
Warren, Dr. Herbert S., 610 Montgomery Avenue, Bryn Mawr, Pennsylvania
Young, Dr. B. P., Cornell University, Ithaca, New York

2. Regular Members

Abell, Dr. Richard G., 7 Cooper Road, New York City, New York

Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts

Addison, Dr. W. H. F., 286 East Sidney Avenue, Mount Vernon, New York

Adolph, Dr. Edward F., University of Rochester, School of Medicine and Dentistry, Rochester, New York

Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota

Allen, Dr. M. Jean, Department of Biology, Wilson College, Chambersburg, Pennsylvania

Allen, Dr. Robert D., Department of Biology, Princeton University, Princeton, New Jersey

Alscher, Dr. Ruth, Department of Physiology, Manhattanville College, Purchase, New York

Amberson, Dr. William R., Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland

Anderson, Dr. J. M., Department of Zoology, Cornell University, Ithaca, New York

Anderson, Dr. Rubert S., Medical Laboratories, Army Chemical Center, Maryland (Box 632 Edgewood, Maryland)

Anderson, Dr. T. F., c/o Dr. A. Lurff, Institut Pasteur, 28 Rue du Dr. Roux, Paris 15e, France

Armstrong, Dr. Philip B., State University of New York College of Medicine, Syracuse 10, New York

Arnold, Dr. William A., Oak Ridge National Laboratory, Oak Ridge, Tennessee

Atwood, Dr. Kimball C., 68½ Outer Drive, Oak Ridge, Tennessee

Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts

Ayers, Dr. John C., Department of Zoology, University of Michigan, Ann Arbor, Michigan

Baitsell, Dr. George A., Osborn Zoological Laboratories, Yale University, New Haven, Connecticut

Baker, Dr. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania

Ball, Dr. Eric G., Department of Biological Chemistry, Harvard University Medical School, Boston 15, Massachusetts

Bang, Dr. F. B., Department of Pathobiology, Johns Hopkins University School of Hygiene, Baltimore 5, Maryland

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire

Bard, Dr. Philip, Johns Hopkins Medical School, Baltimore, Maryland

Barth, Dr. L. G., Department of Zoology, Columbia University, New York City, New York

Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois

Beams, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa

Beck, Dr. L. V., Department of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania

Beers, Dr. C. D., University of North Carolina, Chapel Hill, North Carolina

Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana
Benesch, Dr. Reinhold, Marine Biological Laboratory, Woods Hole, Massachusetts
Benesch, Dr. Ruth, Marine Biological Laboratory, Woods Hole, Massachusetts
Bennett, Dr. Miriam, Department of Biology, Sweet Briar College, Sweet Briar, Virginia
Berg, Dr. William E., Department of Zoology, University of California, Berkeley, California
Berman, Mr. Mones, Sloan-Kettering Institute, 410 E. 68th Street, New York 21, New York
Bernstein, Dr. Maurice, Virus Laboratory, University of California, Berkeley 4, California
Bernheimer, Dr. Alan W., New York University College of Medicine, New York 16, New York
Bertholf, Dr. Floyd M., College of the Pacific, Stockton, California
Bevelander, Dr. Gerrit, New York University School of Medicine, New York 16, New York
Bigelow, Dr. Henry B., Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts
Bishop, Dr. David W., Department of Embryology, Carnegie Institution of Washington, Baltimore 5, Maryland
Blanchard, Dr. K. C., Johns Hopkins Medical School, Baltimore, Maryland
Bloch, Dr. Robert, c/o Biological Abstracts, 3815 Walnut Street, Philadelphia 4, Pennsylvania
Blum, Dr. Harold F., 24 Rue de Babylone, Paris VII, France
Bodansky, Dr. Oscar, Department of Biochemistry, Memorial Cancer Center, 444 East 68th Street, New York 21, New York
Bodian, Dr. David, Department of Epidemiology, Johns Hopkins University, Baltimore 5, Maryland
Boell, Dr. Edgar J., Yale University, New Haven, Connecticut
Boettiger, Dr. Edward G., Department of Zoology, University of Connecticut, Storrs, Connecticut
Bold, Dr. Harold C., Department of Botany, University of Texas, Austin, Texas
Borei, Dr. Hans, Department of Zoology, University of Pennsylvania, Philadelphia, Pennsylvania
Bowen, Dr. Vaughan T., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts
Bradley, Dr. Harold C., 2639 Durant Avenue, Berkeley 4, California
Bridgman, Dr. Anna J., Department of Biology, Agnes Scott College, Decatur, Georgia
Bronk, Dr. Detlev W., Rockefeller Institute, 66th Street & York Avenue, New York 21, New York
Brooks, Dr. Matilda M., Department of Physiology, University of California, Berkeley 4, California
Brown, Dr. Dugald E. S., Department of Zoology, University of Michigan, Ann Arbor, Michigan
Brown, Dr. Frank A., Jr., Department of Biological Sciences, Northwestern University, Evanston, Illinois

Brownell, Dr. Katherine A., Ohio State University, Columbus, Ohio
Buck, Dr. John B., Laboratory of Physical Biology, National Institutes of Health, Bethesda, Maryland (10405 Muir Place, Kensington, Maryland)
Bullington, Dr. W. E., Randolph-Macon College, Ashland, Virginia
Bullock, Dr. T. H., Department of Zoology, University of California, Los Angeles 24, California
Burbanck, Dr. William D., Box 834, Emory University, Georgia
Burdick, Dr. C. Lalor, The Lalor Foundation, 4400 Lancaster Pike, Wilmington, Delaware
Burkenroad, Dr. M. D., c/o Lab. Nal. de Pesca, Apartado 3318, Estofeta #1, Olindania, Republic of Panama
Butler, Dr. E. G., Department of Biology, Princeton University, Princeton, New Jersey
Cameron, Dr. J. A., Baylor College of Dentistry, Dallas, Texas
Cantoni, Dr. Giulio, National Institutes of Health, Mental Health, Bethesda 14, Maryland
Carlson, Dr. Francis D., Department of Biophysics, Johns Hopkins University, Baltimore, Md.
Carpenter, Dr. Russell L., Tufts College, Medford 55, Massachusetts
Carson, Miss Rachel, 204 Williamsburg Drive, Silver Spring, Maryland
Cattell, Dr. McKeen, Cornell University Medical College, 1300 York Avenue, New York City, New York
Cattell, Mr. Ware, Cosmos Club, Washington 5, D. C.
Chaet, Dr. Alfred B., Boston University School of Medicine, 80 E. Concord Street, Boston 18, Massachusetts
Chambers, Dr. Edward, Department of Physiology, University of Miami Medical School, Coral Gables, Florida
Chang, Dr. Joseph J., National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland
Chase, Dr. Aurin M., Department of Biology, Princeton University, Princeton, New Jersey
Cheney, Dr. Ralph H., Biology Department, Brooklyn College, Brooklyn 10, New York
Claff, Mr. C. Lloyd, 5 Van Beal Road, Randolph, Massachusetts
Clark, Dr. A. M., Department of Biological Sciences, University of Delaware, Newark, Delaware
Clark, Dr. E. R., The Wistar Institute, Woodland Avenue and 36th Street, Philadelphia 4, Pennsylvania
Clark, Dr. Leonard B., Department of Biology, Union College, Schenectady, New York
Clarke, Dr. George L., Harvard University, Biological Laboratory, Cambridge 38, Massachusetts
Cleland, Dr. Ralph E., Indiana University, Bloomington, Indiana
Clement, Dr. A. C., Department of Biology, Emory University, Emory, Georgia
Clowes, Dr. G. H. A., Eli Lilly and Company, Indianapolis, Indiana
Coe, Dr. W. R., 183 Third Avenue, Chula Vista, California
Cohen, Dr. Seymour S., Department of Physiological Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania

Cole, Dr. Kenneth S., National Institutes of Health (NINDB), Bethesda 14, Maryland
Collett, Dr. Mary E., 34 Weston Road, Wellesley 81, Massachusetts
Collier, Dr. Jack R., Department of Zoology, Louisiana State University, Baton Rouge, Louisiana
Colton, Dr. H. S., Box 601, Flagstaff, Arizona
Colwin, Dr. Arthur L., Department of Biology, Queens College, Flushing, New York
Colwin, Dr. Laura H., Department of Biology, Queens College, Flushing, New York
Cooperstein, Dr. Sherwin J., Department of Anatomy, Western Reserve University Medical School, Cleveland, Ohio
Copeland, Dr. D. E., 1027 N. Manchester Street, Arlington 5, Virginia
Copeland, Dr. Manton, Bowdoin College, Brunswick, Maine
Copley, Dr. Alfred L., Centre National de Transfusion Sanguine, 6, Rue Alexandra-Cobonel, Paris XVe, France
Cornman, Dr. Ivor, Hazleton Laboratories, Box 333, Falls Church, Virginia
Costello, Dr. Donald P., Department of Zoology, University of North Carolina, Chapel Hill, North Carolina
Costello, Dr. Helen Miller, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina
Crane, Mr. John O., Woods Hole, Massachusetts
Croasdale, Dr. Hannah T., Dartmouth College, Hanover, New Hampshire
Crouse, Dr. Helen V., Goucher College, Baltimore, Maryland
Crowell, Dr. P. S., Jr., Department of Zoology, Indiana University, Bloomington, Indiana
Csapo, Dr. Arpad I., Rockefeller Institute for Medical Research, New York 21, New York
Curtis, Dr. Maynie R., University of Miami, Box 1015, South Miami, Florida
Curtis, Dr. W. C., University of Missouri, Columbia, Missouri
Dan, Dr. Jean Clark, Misaki Biological Station, Misaki, Japan
Dan, Dr. Katsuma, Misaki Biological Station, Misaki, Japan
Danielli, Dr. James F., Department of Zoology, King's College, London, England
Davis, Dr. Bernard D., Department of Pharmacology, New York University College of Medicine, New York 16, New York
Dawson, Dr. A. B., Harvard University, Cambridge 38, Massachusetts
Dawson, Dr. J. A., College of the City of New York, New York City, New York
Deane, Dr. Helen W., Albert Einstein College of Medicine, New York 61, New York
Diller, Dr. Irene C., Institute for Cancer Research, Philadelphia, Pennsylvania
Diller, Dr. William F., 2417 Fairhill Avenue, Glenside, Pennsylvania
Dixon, Dr. Frank J., Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania
Dodds, Dr. G. S., West Virginia University School of Medicine, Morgantown, West Virginia
Dolley, Dr. William L., Department of Biology, Randolph-Macon College, Ashland, Virginia

Donaldson, Dr. John C., University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania
Doty, Dr. Maxwell S., Department of Biology, University of Hawaii, Honolulu, T. H.
DuBois, Dr. Eugene F., 200 East End Avenue, New York 28, New York
Duryee, Dr. William R., George Washington University School of Medicine, Department of Physiology, Washington 5, D. C.
Edds, Dr. Mac V., Jr., Department of Biology, Brown University, Providence 12, Rhode Island
Edwards, Dr. Charles, Wilmer 25, Johns Hopkins Hospital, Baltimore 5, Maryland
Eichel, Dr. Bertram, Bureau of Biological Research, Box 515, Rutgers University, New Brunswick, New Jersey
Eichel, Dr. Herbert J., Hahnemann Medical College, Philadelphia, Pennsylvania
Elliott, Dr. Alfred M., Department of Zoology, University of Michigan, Ann Arbor, Michigan
Evans, Dr. Titus C., State University of Iowa, Iowa City, Iowa
Failla, Dr. G., College of Physicians and Surgeons, Columbia University, New York City, New York
Fauré-Fremiet, Dr. Emmanuel, Collège de France, Paris, France
Ferguson, Dr. F. P., Department of Physiology, University of Maryland Medical School, Baltimore 1, Maryland
Ferguson, Dr. James K. W., Connought Laboratories, University of Toronto, Ontario, Canada
Figge, Dr. F. H. J., University of Maryland Medical School, Lombard and Green Streets, Baltimore 1, Maryland
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BACON, MRS. ROBERT
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BARB, MR. ROBERT P.
BARTOW, MR. AND MRS. CLARENCE
BARTOW, MRS. FRANCIS D.
BARTOW, MR. AND MRS. PHILIP
BELL, MRS. ARTHUR
BRADLEY, MR. ALBERT L.
BRADLEY, MRS. CHARLES CRANE
BROWN, MRS. THORNTON
BURLINGAME, MRS. F. A.
CAHOON, MRS. SAMUEL
CALKINS, MR. G. NATHAN, JR.
CALKINS, MRS. GARY N.
CARLETON, MRS. WINSLOW
CLAFF, MR. AND MRS. C. LLOYD
CLARK, DR. AND MRS. ALFRED HULL
CLARK, MRS. LEROY
CLARK, MR. W. VAN ALAN
CLOWES, MR. ALLEN W.
CLOWES, MRS. G. H. A.
CLOWES, DR. AND MRS. GEORGE, JR.
COLTON, MR. H. SEYMOUR
CRANE, MISS LOUISE
CRANE, MRS. W. CAREY
CRANE, MRS. W. MURRAY
CROWELL, MR. PRINCE S.
CURTIS, DR. W. D.
DANIELS, MR. AND MRS. F. HAROLD
DAY, MR. AND MRS. POMEROY
DRAPER, MRS. MARY C.
DREYER, MRS. FRANK
ELSMITH, MRS. DOROTHY
ENDERS, MR. FREDERICK
EWING, MR. FREDERICK
FASEY, MRS. PAULINE M.
FAY, MRS. BRUCE CRANE
FRANCIS, MR. LEWIS, JR.
FROST, MRS. EUGENIA

Galtsoff, Mrs. Eugenia
Gifford, Mr. and Mrs. John A.
Gildea, Dr. and Mrs. E. F.
Green, Miss Gladys W.
Hamlen, Mr. J. Monroe
Harrell, Mr. and Mrs. Joel E.
Harrington, Mr. and Mrs. A. W.
Harrington, Mr. Robert D.
Hirschfeld, Mrs. Nathan
Houston, Mr. and Mrs. Howard E.
Jewett, Mrs. George F.
Keith, Mr. and Mrs. Harold C.
King, Mr. Franklin
Koller, Mrs. Lewis
Lemann, Mrs. Solen B.
Lobb, Mrs. John
Lurdon, Mr. W. R.
McKeloy, Mr. John
Marvin, Mrs. Walter T.
Mast, Mrs. S. O.
Meigs, Mrs. Edward B.
Meigs, Dr. and Mrs. J. Wister
Mitchell, Mrs. James McC.
Mixter, Mrs. Jason
Mosser, Mrs. Florence M.
Motley, Mrs. Thomas
Newton, Miss Helen K.
Nichols, Mrs. George
Nims, Mrs. E. D.
Packard, Dr. and Mrs. Charles
Packard, Mrs. Laurence B.
Park, Mr. Malcolm S.
Pennington, Miss Anne H.
Redfield, Mrs. Alfred
Reznikoff, Dr. Paul
Riggs, Mrs. Lawrason
Rivinus, Mr. and Mrs. F. Markoe
Root, Mrs. Walter
Rozendoal, Mr. H. M.
Rudd, Mrs. H. W. Dwight
Sands, Miss Adelaide G.
Saunders, Mrs. Lawrence
Shiverick, Mrs. Mary
Stone, Mr. and Mrs. S. M.
Swift, Mr. and Mrs. E. Kent
Swope, Mr. and Mrs. Gerard, Jr.
Swope, Miss Henrietta H.
Tilney, Mrs. Albert A.
Tompkins, Mr. and Mrs. B. A.
Webster, Mrs. Edwin S.
Whitely, Miss Mabel W.
Wickersham, Mr. and Mrs. James H.
Williston, Miss Emily
Wolfinsohn, Mrs. Wolfe

V. REPORT OF THE LIBRARIAN

In 1957, seventy-six new journals were acquired, bringing the total number of currently received titles to 1635. Of these titles, there were 490 (15 new) Marine Biological Laboratory subscriptions; 617 (14 new) exchanges and 192 (21 new) gifts; 90 (9 new) were Woods Hole Oceanographic Institution subscriptions; 191 (7 new) were exchanges and 55 (10 new) were gifts. During the past ten years, we averaged 60 new journals per year. The ever growing number of new journals being issued far exceeds the number which cease publication.

The Laboratory purchased 151 books, received 61 complimentary copies (4 from authors and 57 from publishers), and accepted 13 miscellaneous gifts. The Institution purchased 39 titles and received 10 gifts. The total number of books accessioned amounted to 274.

By purchase and by gift the Laboratory completed 13 journal sets and partially completed 19. The Institution completed 4 sets and partially completed 3. There were 3920 reprints added to the collection, of which 2055 were of current issue.

At the close of the year, the Library contained 67,961 bound volumes and 206,125 reprints.

The Library sent out on inter-library loan 243 volumes and borrowed 115 for the convenience of the scientists. It is hoped that a copying machine may be purchased in the near future so that short papers may be reproduced for out-of-town loans, thus eliminating some of the depreciation on our volumes. A process such as this could also be utilized for summer service.

Reprint collections were received from the estate of Dr. Arthur Weysse and from the University of Pittsburgh; many books, journal numbers and papers were received from Drs. Ethel B. Harvey, C. Ladd Prosser, Rufus R. Humphrey, Phineas W. Whiting, Paul S. Galtsoff, Ralph Wichterman, and the Tompkins-McCaw Library, Medical College of Virginia. Dr. Alfred W. Senft kindly donated back volumes and a current subscription to the "New England Journal of Medicine." Grateful acknowledgment is herewith extended to the donors of these very acceptable presentations.

With a larger sum available for the purchase of books, and with the many suggestions so willingly submitted by the Library Advisory Committee, we were in a position in 1957 to add many new titles to the shelves. An increase in the binding budget also enabled us to have bound 275 back volumes, bringing the total to 1110 for the year. This same degree of progress is anticipated in 1958.

Respectfully submitted,

DEBORAH L. HARLOW,
Librarian

VI. REPORT OF THE TREASURER

The market value of both the General Fund and the Library at December 31, 1957, amounted to $1,461,278 as compared with the total of $1,472,265 as of December 31, 1956. The average yield on the securities was 3.84% of market value and 5.60% of book value. The total uninvested principal cash in the above accounts as of December 31, 1957, was $2,248. Classification of the securities held in the Endowment Funds appears in the auditor's report.

The market value of the pooled securities as of December 31, 1957, was $247,629 with uninvested principal cash of $102. The book value of the securities in this account was $236,735. The average yield on market value was 3.88% and 4.06% of book value.

The proportionate interest in the Pooled Fund account of the various Funds as of December 31, 1957, is as follows:

Pension Fund	17.608%
General Laboratory Investment	57.866
Other:	
Bio Club Scholarship Fund	1.687
Rev. Arsenious Boyer Scholarship Fund	2.064
Gary N. Calkins Fund	1.933
Allen R. Memhard Fund	.374
F. R. Lillie Memorial Fund	6.515

Lucretia Crocker Fund	7.054
E. G. Conklin Fund	1.194
M. H. Jacobs Scholarship Fund	.850
Jewett Memorial Fund	.626
Anonymous Gift	2.229

The Pooled Fund includes the Jewett Memorial Fund and an anonymous Gift Fund which were additions during 1957. The Jewett Memorial Fund was created by gifts in memory of the late George Frederick Jewett. Mr. Jewett as well as his father and mother and the other members of his family have been keenly interested in the Laboratory since its inception. It has not yet been determined how the Jewett Fund and the fund created by the anonymous gift will be used, but the views of the Jewett family and the donor of the latter fund will be given first consideration.

Considerable activity was recorded in the special custodian account owing to the purchase of short-term Government bonds to activate available cash which would otherwise remain idle in our regular cash accounts pending payment of construction expenses. Income earned was $646.40.

Inasmuch as the MBL Club loan was reduced to $2,052, the securities pledge to cover this loan was reduced to $3,000.

Donations from MBL Associates for 1957 were $3,481 as compared with $5,255 in 1956. Unrestricted gifts from foundations, societies and companies amounted to $33,000.

For the rehabilitation of the Crane Building, the National Science Foundation advanced $415,000 in 1957. Construction began in September and is scheduled for completion in May of 1958.

In April of 1957 we paid off the David House Mortgage in the amount of $5,000.

Lynbrand, Ross Bros. & Montgomery have examined our books and submitted financial statements for examination.

Following is a statement of the auditors.

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of Marine Biological Laboratory as at December 31, 1957, the related statements of operating expenditures and income for the year then ended, and statement of current fund for the year ended December 31, 1957. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1957, and the expenditures and income for the year then ended.

LYBRAND, ROSS BROS. & MONTGOMERY

Boston, Massachusetts
May 22, 1958

JAMES H. WICKERSHAM,
Treasurer

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1957

Investments

Investments held by Trustee:	
Securities, at cost (approximate market quotation $1,461,278)	$1,002,682
Cash	2,248
	1,004,930
Investments of other endowment and unrestricted funds:	
Pooled Investments, at cost (approximate market quotation $247,629)	236,735
Less temporary investment of current fund cash	5,728
	231,007
Other investments (Note A)	67,323
Cash	11,263
Accounts receivable	5,038
	314,631

Plant Assets

Land, buildings, library and equipment (Note B)	2,517,845
Less allowance for depreciation (Note B)	1,026,681
	1,491,164
Construction in progress	103,856
Cash	34,560
U. S. Treasury bills, due 1/30/58, at cost (face value $350,000)	346,815
	1,976,395

Current Assets

Cash	142,160
U. S. Treasury bills, at cost:	
$40,000 face value due 2/13/58	39,649
Temporary investment in pooled securities	5,728
Accounts receivable (U. S. Government $19,605)	36,274
Inventories of specimens and Bulletins	57,282
Prepaid insurance and other	13,531
	$3,590,580

Notes:

A—The Laboratory has guaranteed a note of approximately $2,400 of the M.B.L. Club and has pledged as security therefor bonds with an original cost of $3,000 included in other investments.

B—The Laboratory has since January 1, 1916, provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

Balance Sheet

December 31, 1957

Endowment Funds

Endowment funds given in trust for benefit of the Marine Biological Laboratory		$1,004,930
Endowment funds for awards and scholarships:		
Principal	$ 64,415	
Unexpended income	2,428	66,843
Unrestricted funds functioning as endowment		206,378
Retirement fund		46,233
Pooled investments—accumulated gain or (loss)		(4,823)
		314,631

Plant Liability and Funds

Funds expended for plant, less retirements	$2,551,469	
Less allowance for depreciation charged thereto	1,026,681	1,524,788
Unexpended plant funds		381,375
		1,906,163
Accounts payable		70,232
		1,976,395

Current Liabilities and Funds

Accounts payable	43,409
Unexpended balances of gifts for designated purposes	8,744
Advance payments on research contracts	94,217
Current fund	148,254
	$3,590,580

MARINE BIOLOGICAL LABORATORY

Statement of Operating Expenditures and Income

Year Ended December 31, 1957

Operating Expenditures

Direct expenditures of departments:	
Research and accessory services	$146,859
Instruction	35,237
Library, including book purchases	32,712
Biological Bulletin	16,995
	231,803
Direct costs on research contracts	129,983
Administration and general	54,526
Plant operation and maintenance	81,156
Dormitories and dining services	143,322
Plant additions from current funds	59,581
	700,371
Less depreciation included in plant operation and dormitories and dining services above but charged to plant funds	36,351
	664,020

Income

Direct income of departments:	
Research fees	43,418
Accessory services (including sales of biological specimens $67,562)	103,718
Instruction fees	16,980
Library fees and income	8,239
Biological Bulletin, subscriptions and sales	19,846
	192,201
Reimbursement and allowance for direct and indirect costs on research contracts	151,444
Dormitories and dining services income	108,349
	451,994
Investment income used for current expenses:	
Endowment funds	83,984
Current fund investments	1,645
Gifts used for current expenses	127,301
Sundry income	175
Total current income	665,099
Excess of income	1,079

MARINE BIOLOGICAL LABORATORY

Statement of Current Fund

Year Ended December 31, 1957

Balance January 1, 1957	$147,175
Excess of income over operating expenditures 1957	1,079
Balance December 31, 1957	$148,254

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1957

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1957
Securities held by Trustee:					
General endowment fund:					
U. S. Government bonds	$ 81,000	9.7	$ 81,000	6.8	$ 2,359
Other bonds	420,980	50.2	403,589	33.7	12,246
	501,980	59.9	484,589	40.5	14,605
Preferred stocks	85,788	10.2	71,713	6.0	3,370
Common stocks	251,097	29.9	641,355	53.5	28,312
	838,865	100.0	1,197,657	100.0	46,287
General Educational Board endowment fund:					
U. S. Government bonds	25,000	15.3	25,000	9.5	749
Other bonds	70,530	43.0	68,813	26.1	2,327
	95,530	58.3	93,813	35.6	3,076
Preferred stocks	27,281	16.7	24,337	9.2	1,130
Common stocks	41,006	25.0	145,471	55.2	5,608
	163,817	100.0	263,621	100.0	9,814
Total securities held by Trustee	$1,002,682		$1,461,278		$56,101
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government bonds	—	—	—	—	833
Other bonds	138,302	58.4	141,416	57.1	3,105
	138,302	58.4	141,416	57.1	3,938
Common stocks	98,433	41.6	106,213	42.9	5,676
	236,735	100.0	$ 247,629	100.0	9,614
Other investments:					
U. S. Government bonds	2,970				131
Common stocks	43,600				23,444
Real estate and mortgage	20,753				—
	67,323				23,575
Total investments of other endowment and unrestricted funds	$ 304,058				$33,189
Total investment income					89,290
Custodian's fee charged thereto					(574)
Income of current funds temporarily invested in pooled securities					(204)
Investment income distributed to funds					$88,512

COELOMIC CORPUSCLES OF ECHINODERMS[1]

RICHARD A. BOOLOOTIAN[2] AND ARTHUR C. GIESE

Hopkins Marine Station of Stanford University, Pacific Grove, California

Although a variety of corpuscles have been described during the last century by investigators of echinoderm perivisceral fluid, disagreement exists among the descriptions of different authors and a re-investigation of the problem with newer methods is desirable before the corpuscles of echinoderm perivisceral fluid can be properly characterized. These newer methods are primarily observation through the phase contrast microscope, so effective in Grégoire's studies (1953) on insect blood, and observation of cells unaltered by contact with air, glass or chemicals which Hensill (1949) found so useful in his study of crab blood. In addition, the study of all the transformations of a cell of a given type under gradually altered conditions discloses changes from one cell type to another in some instances. Furthermore, a comparative study made possible a useful tentative classification of the cells found in fifteen species of echinoderms representing all the living classes of Echinodermata.

Materials and Methods

The animals were collected in the vicinity of the Monterey Peninsula at low tide in some cases and by dredging in others. The animals were used as soon after collection as possible since starvation is known to alter clotting (Glavind, 1948). Cell types of each species were determined by the examination of fluid drawn from the perivisceral cavity with the aid of a siliconized syringe. A drop of the fluid was placed on a siliconized cover slip which was inverted over a depression slide, and examined immediately at magnifications of 43 × and 97 × and photographed periodically.

The optical equipment consisted of a Spencer 18 ML phase microscope equipped with a Spencer phase turret condenser, bright contrast objectives and wide field oculars. The source of illumination was an Ortho-Illuminator-B (American Optical Co.), using 100–300 watt bulbs.

The photomicrographic equipment used was a Kine-Exacta model VX camera coupled to a Leitz Micro-Ibso attachment. Exposures were made on Microfile film which was developed in D-11 developer and printed on single weight glossy surface DuPont Varigram paper.

Since contact with air is known to alter the morphology of cells, the perivisceral fluid was taken up into evacuated capillaries. The capillaries were prepared by

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[1] Supported in part by National Science Foundation Grant GS-482 and Public Health Grant RG-4578 (C).

We are also indebted to Dr. A. R. Moore for his sustained interest and suggestions, to Dr. L. Blinks for accommodations and suggestions, to Dr. R. L. Bolin for extending use of facilities and for helpful criticism, and to Mr. A. Farmanfarmaian for counsel and advice.

[2] Now at the Department of Zoology, University of California at Los Angeles.

pulling 5-mm. Pyrex tubing in such a manner that the capillary diameters never exceeded 1 mm.

The inner walls of the capillaries were coated with silicon (G.E. Dri-Film) by aspirating the reagent and subsequent drying. They were then flame-sealed at one end, evacuated, and flame-sealed at the other end in 7.5-cm. segments. Silicon was used because it coats the glass and prevents cytolysis of cells coming in contact with clean glass (Jacques *et al.*, 1946). Each capillary was scratched with a carborundum point half a centimeter from one end. The scratched end inserted through the peristome (echinoids) or a dermal branchia (asteroid) can be broken at the scratch by a slight pressure, and the body fluid is aspirated into the capillary. In the case of holothuroids a longer capillary, scratched in the center, was inserted into the interambulacral margin of the animal and broken in the middle in the same manner. The open tip of the capillary was covered with silicon grease upon removal. Then the capillary was placed on a slide in a channel filled with glycerine and covered with a cover slip. With this method it is possible to study types for at least five minutes before clotting appears, and to observe any changes which occur during this time. Furthermore, the capillary tubes can be rotated and the nature of the corpuscles ascertained in three dimensions. Clots also can be studied effectively in such preparations. This method readily lends itself to photography.

In order to determine which coelomic cells, if any, were phagocytic, one ml. of finely ground carmine suspension in sea water was injected by a syringe through the peristomial membrane in echinoids, through a dermal branchia in asteroids and through the body wall in holothuroids. At various time intervals, ranging from ten minutes to five days, hanging drop and capillary-tube preparations of the perivisceral fluid were examined and photographed.

Classification of Corpuscles of Echinoderm Body Fluids

The results of the present study, documented in succeeding sections, revealed thirteen types of fairly distinct cells (see Tables I and II). Some of these corpuscles appear to be phyletic in distribution, *e.g.*, the bladder amebocytes (Fig. 1) and the filiform amebocytes (Fig. 2), the first of which occur in thirteen of the species examined and the latter in twelve of the species examined. As will be discussed later, these two cell types are different phases of the same cell, e.g., in *Pisaster ochraceus*. The small spherical amebocytes (Fig. 3) are found in three of the asteroids investigated and in the ophiuroid and the crinoid. The fusiform corpuscle (Fig. 4), the vibratile corpuscle (Fig. 6), the eleocyte (Fig. 7), and hyaline hemocyte (Fig. 8) are found in the sea urchins only. The colorless spherical amebocyte (Fig. 5) is common both to the sea urchins and sand dollars. The other types of cells have a rather limited distribution. The large spherical corpuscle (Fig. 9) and the red corpuscle (Fig. 10) are found in the sand dollar and the crinoid. The lobular corpuscle (Fig. 11), on the other hand, is limited to the crinoid only. The hyaline plasma amebocyte (Fig. 12) is found in the starfish *Poraniopsis*. Cells "staining" with osmic acid (Fig. 13) are observed only in the sand dollar.

Corpuscles of Asteroids

The fluid within the spacious coelomic cavity of the asteroids contains coelomocytes of fewer types than occur in other classes. Two main types of cells have been

TABLE I

Distribution of corpuscular elements

Species	Bladder amebocyte	Filiform amebocyte	Small spherical corpuscle	Fusiform corpuscle	Colorless spherule amebocyte	Vibratile corpuscle	Eleocyte amebocyte	Hyaline hemocyte	Large spherical corpuscle	Red corpuscle	Lobular corpuscle	Hyaline plasma amebocyte	Cells "*staining*" with osmic acid
Astropecten californicus	x	x											
Mediaster aequalis	x	x	x										
Poranioposis inflata	x	x	x									x	
Patiria miniata	x	x											
Pycnopodia helianthoides	x	x	x										
Pisaster ochraceus	x	x											
Pisaster giganteus	x	x											
Pisaster brevispinus	x	x											
Strongylocentrotus purpuratus	x	x		x	x	x	x	x					
Strongylocentrotus franciscanus	x	x		x	x	x	x	x					
Strongylocentrotus fragilis	x	x		x	x	x	x	x					
Dendraster excentricus	x				x				x	x			x
Gorgonocephalus eucnemis			x	x									
Heliometra glacialis			x	x					x	x	x		
Stichopus californicus	x	x			x	x							
Figure number	1	2	3	4	5	6	7	8	9	10	11	12	13

TABLE II

Properties of coelomic corpuscles

Cell type	Range or size in μ*	Color	Granules	Vacuoles	Function	Citation
Bladder amebocyte	9–51	colorless, gray	numerous black	several	phagocytic	Kindred, 1921
Filiform amebocyte	8–55	gray	several black	two–many	clot, phagocytic	Kindred, 1921
Small spherical amebocyte	4–8, 7–35	green, yellow, red	black and red	occasional	clot	Cuénot, 1888
Fusiform corpuscle	2–12 × 6–30	gray	0	0	?	Cuénot, 1891
Colorless spherical amebocyte	8–12 × 13.6–28	pale yellow	lobular	0	lipid transport?	Kindred, 1921
Vibratile corpuscle	3–11.7 × 9–44	gray	numerous black	numerous small	circulation?	Kindred, 1921
Eleocyte	11.2–29 × 6.8–8	red	small red	0	O_2 transport?	MacMunn, 1885
Hyaline hemocyte	9.2–13	pale yellow	0	numerous	clot	This paper
Large spherical corpuscle	19–25.6	slightly brownish	brown	several large	?	Bookhout *et al.*, 1940
Red corpuscle	5.5–11.2 × 8–16	red	0	0	?	This paper
Lobular corpuscle	16–27 × 19–28.8	gray	0	0	?	Cuénot, 1891
Hyaline plasma amebocyte	17.04–31.2	gray	black	several	?	This paper
Osmophilic cells	10–12.3	pale yellow	0	0	?	This paper

* Length or length and width. A wide range is observed in some cases because the same type of corpuscle is of a different size in different species in which it is found.

described by Théel (1919), Kindred (1924), Lison (1930), Goodrich (1919), Durham (1888), Geddes (1879, 1880), Cuénot (1891): amebocytes with ordinary slender pseudopodia (filiform amebocytes) and amebocytes with petaloid or bladder extrusions called pseudopodia (bladder amebocytes).

In the present study both bladder amebocytes (Fig. 1) and filiform amebocytes (Fig. 2) were found to be abundant in all species of asteroids investigated. Another cell type, a small spherical corpuscle with pigmented granules, however, was also found in *Pycnopodia helianthoides*, *Mediaster aequalis,* and *Poraniopsis inflata.* The cells of the first two species contain a light red or orange intracellular pigment which is particularly obvious when the cells are concentrated by centrifugation. In *Poraniopsis inflata* the pigmented corpuscles contain black granules, but not in conspicuous quantity.

CORPUSCLES OF ECHINOIDS

The body fluid of the echinoids contains several types of cells which have been studied most extensively by Geddes (1879), Cuénot (1891), Théel (1896), Kindred (1921), Behre (1932), Boliek (1935), Kuhl (1937), Bookhout and Greenburg (1940), Liebman (1950), and Schinke (1950). Six types of cells have been described by various workers: amebocytes with spiked pseudopodia, amebocytes with petaloid pseudopodia, colorless spherule amebocytes, greenish and yellowish spherule amebocytes, red spherule amebocytes, and vibratile corpuscles which are small spherical cells provided with a flagellum.

Seven types of cells were identified in the body fluid of the echinoids investigated here: bladder amebocytes, filiform amebocytes, fusiform corpuscles, colorless

Plate I

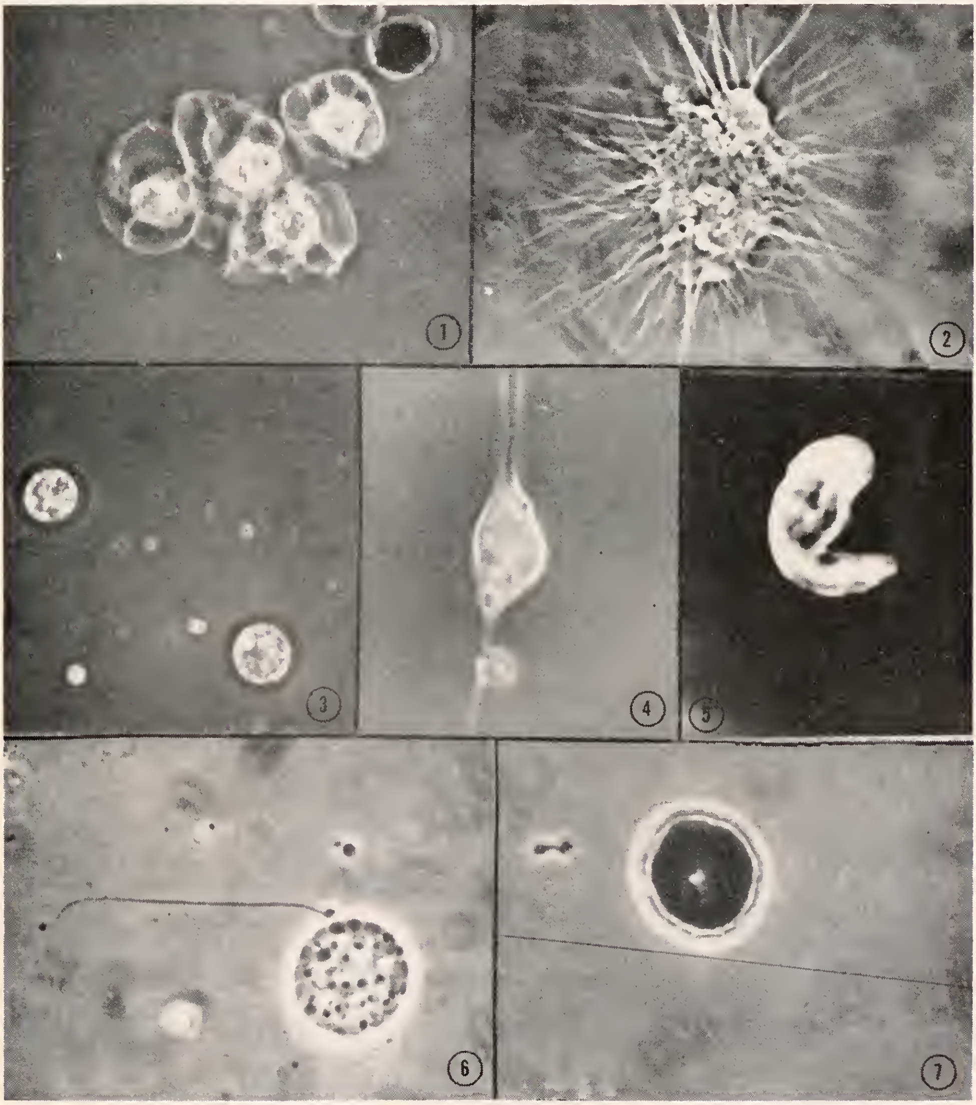

Figure 1, bladder amebocytes of *Dendraster excentricus.* Figure 2, filiform amebocyte of *Pisaster ochraceus.* Figure 3, small spherical corpuscle of *Heliometra glacialis.* Figure 4, fusiform corpuscle of *Strongylocentrotus franciscanus.* Figure 5, colorless spherule amebocyte of *Strongylocentrotus purpuratus.* Figure 6, vibratile corpuscle of *Strongylocentrotus purpuratus.* Figure 7, eleocyte of *Strongylocentrotus franciscanus.*

Photomicrographs were taken at a magnification of 344 × and were enlarged subsequently.

Plate II

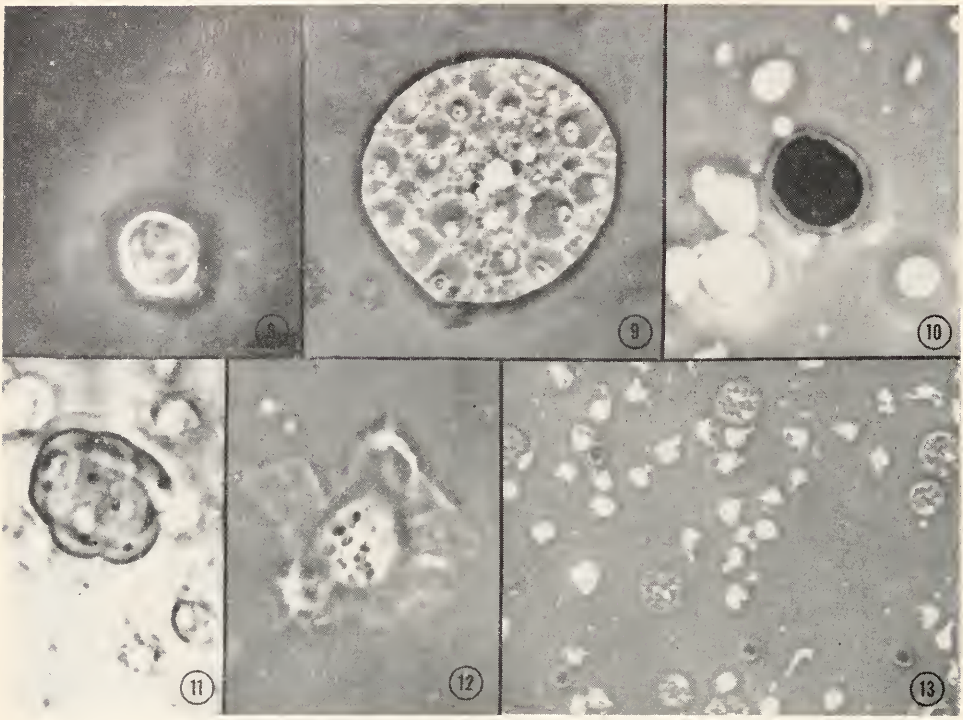

Figure 8, hyaline hemocyte of *Strongylocentrotus purpuratus*. Figure 9, large spherical corpuscle of *Dendraster excentricus*. Figure 10, red corpuscle of *Heliometra glacialis*. Figure **11**, lobular corpuscle of *Heliometra glacialis*. Figure 12, hyaline plasma amebocyte of *Poraniopsis inflata*. Figure 13, osmophilic cells of *Dendraster excentricus* (small dark oval units).

Photomicrographs were taken at a magnification of 344 × and were enlarged subsequently.

spherule amebocytes, vibratile corpuscles, eleocytes, and hyaline hemocytes (Figs. 1, 2, 4, 5, 6, 7, 8).

The bladder amebocytes with large ectoplasmic extrusions and the filiform amebocytes are phagocytic. The eleocytes contain red spherules of echinochrome (MacMunn, 1885; Kuhn and Wallenfels, 1939), a common echinoid pigment which has been demonstrated in the three species of *Strongylocentrotus*. The colorless spherule amebocytes with spherical inclusions lack phagocytic power and move by extending broad round eruptive (guttata) pseudopodia. The fusiform corpuscles, which are few in number, have no known function. The hyaline hemocyte gives rise to an extracellular clot after disintegrating at the site of an injury. These disintegrating cells resemble the colorless spherule amebocytes to a considerable degree and, as a result, may have escaped the observations of past investigators because most of their observations were made on fixed and stained material.

The eleocytes have no known function, although several have been suggested by various investigators. Griffiths (1892) concluded that they are associated with oxygen transport as did Awerinzew (1911). Cuénot (1891) opposed this assumption on the basis that no color change was observed when pigment was allowed to stand in air, and proposed that instead of being an oxygen-carrying agent, the eleocyte was a store of food material which the cells had taken from the intestine. The concentration of eleocytes in the body fluids differs in the three species of *Strongylocentrotus* studied. *Strongylocentrotus purpuratus* contains 1100 to 1700, *S. franciscanus,* 500 to 700, and *S. fragilis,* 100 to 150 cells/mm^3. The red cells are found in the epithelial lining of the sea urchin test and contribute to its color.

The vibratile corpuscles (Fig. 6) found in the sea urchins are of uncertain origin and function. They do not participate in clotting, except as they are incidentally caught in the mesh of fibers in the extensive clot formed in sea urchin body fluid. It is possible that the vibratile corpuscles aid in the mixing of the body fluid since the current created peripherally by the ciliated epithelium cannot extend far into the body mass. These cells were concentrated by fractional centrifugation (as were other types of corpuscles) and were kept alive in the body fluid of the urchin, *in vitro,* for many hours, but they were not observed to divide.

Corpuscles of Ophiuroids

The coelomic corpuscles of the ophiuroids have been least studied. Cuénot (1888) observed granular ameboid corpuscles with short spiked pseudopodia in an ophiuroid, and Kindred (1924) observed four types of cells: active leucocytes, passive leucocytes, colorless spherule amebocytes, and vibratile corpuscles in *Ophiopholis aculeata.* In the present study two cell types were observed in *Gorgonocephalus*—small spherical corpuscles (Fig. 3) and fusiform corpuscles (Fig. 4). The latter type of cell was not observed by Cuénot, while the small spherical corpuscle resembles that found by Cuénot. The function of these cells is unknown, although Cuénot has remarked that the ameboid granular cells (small spherical corpuscles) unite by their short pseudopodial tips and anastomose into a network, but he did not consider this to be clot formation.

Corpuscles of Crinoids

The coelomocytes of the crinoids have been described by Cuénot (1891), Hamann (1889), and Reichensperger (1912). Cuénot (1891) observed three kinds of cells, a small finely granular type with short spiked pseudopods, a larger pyriform or fusiform slow-moving cell filled with coarse spherules, and a cell filled with safranophil rods, which, according to Cuénot, was rounded when free in the coelomic fluid, pyriform when migrating through the tissues. Hamann observed numerous wandering ameboid cells in various crinoids and described two types, neither of which resembles Cuénot's forms. Reichensperger also noticed two kinds of coelomocytes in *Antedon:* a phagocytic ameboid form with short spiked pseudopods apparently identical with Cuénot's finely granular type and an oblong cell filled with numerous rods and granules.

In the species studied here, *Heliometra glacialis,* five types of cells were found (Table I), two of which are probably identical with the cells described by Cuénot, Hamann, and Reichensperger. The perivisceral fluid contained an abundance of ameboid cells with short pseudopodia (small spherical corpuscles, Figure 3). The second cell type (fusiform corpuscle, Figure 4), termed "pyriform" by Cuénot, was not pear-shaped in *H. glacialis,* but more spindle-like. Occasional oblong bodies which approached pear-form were observed. The large spherical corpuscles, which lacked pseudopodial extensions, were abundant in the body fluid. The red corpuscles were found in small numbers and have not been reported by other investigators. The lobular corpuscles (Fig. 11), few in number, resemble an embryonic morula stage. The functions of these cells were not determined since the volume of fluid from the few specimens available was too small for extensive studies.

Corpuscles of Holothuroids

The coelomic cells of holothuroids have been described by numerous workers, particularly by Hérouard (1889), Becher (1907), Théel (1921), Kindred (1924), Ohuye (1934, 1936a, 1936b) and Endean (1958). They have been collectively called coelomocytes (Hyman, 1955). The cell types present vary in form, number, and size in different holothuroids, but the following kinds are common throughout the class: hemocytes, phagocytes, colorless spherule amebocytes and filiform amebocytes.

Hemocytes which have been reported for many species contain the pigment hemoglobin, as shown by Howell (1885, 1886), Van der Hyde (1922), Hogben and Van der Lingen (1928), and Kobayashi (1932), on *Thyone, Cucumaria, Paracaudina chilensis,* and *Molpadia roretzii,* respectively. They are not found in *Stichopus californicus,* the species studied here.

Phagocytes are found in all species so far studied. Various names such as cells with elongated pseudopodia (Hérouard), hyaline ameboid corpuscles (Ohuye), and bladder amebocytes (Kindred, 1924) have been applied to them. The term, bladder amebocyte (Fig. 1), is preferable since the large bladder-like projections are readily observable when viewed three-dimensionally.

The colorless spherule amebocytes (Fig. 5) were abundant in *Stichopus californicus.* Hamann (1883) designated these as plasma wandering cells. Cuénot (1891), who identified them as muriform cells, considered the proteinaceous spherules to be food reserves.

The homogeneous amebocytes, which lack inclusions, have been reported by Hamann (1883) and Becher (1907). This type of cell is rare and Hyman (1955) considers it a developmental stage of other cell types. It was not found in *Stichopus.*

Théel, Kawamoto, and Ohuye observed crystal-containing cells in several species of holothuroids. The crystals are in the cytoplasm and are mostly rhomboidal in shape. No crystal-containing cells were observed in *Stichopus.*

A cell type which has not been previously reported by investigators in holothuroids is the filiform amebocyte (Fig. 2). In *Stichopus californicus* these cells are actively involved in clot formation and also exhibit phagocytosis.

Discussion

Many types of coelomic corpuscles have been described by various investigators of echinoderm body fluids, most of whom fixed and stained the cells or used live cells without preventing degenerative changes following contact with glass or air. As a consequence their results were not entirely convincing. In the present study in which pains were taken to avoid the above pitfalls, many of the same cell types were seen. However, more confidence may now be attached to the cell types described by the earlier workers, since their appearance has been checked with live cells under conditions which at least delay changes in cells occurring with clotting or agglutination.

Such coelomic cells as were not seen in the preparation made here, but which have been described by previous workers, may constitute additional cell types since the species used in the present study were not the same as theirs. Only future work using the same species of organism, can resolve this uncertainty. In the special case of the hemocytes—hemoglobin-containing cells of certain holothuroids—no question exists of their reality, even though they were not observed in the species of holothuroid used here (*Stichopus californicus*), since hemocytes have been observed in live specimens and recorded many times by various authors.

Some types of coelomocytes were observed in the species examined here which had not been previously described, *e.g.* the red corpuscles of the sand dollar and the crinoid, and the lobular corpuscles of the crinoid.

The existence of bladder amebocytes need no longer be questioned, even though the bladders appear to be petaloid rather than vesicular in fixed preparations (Goodrich, 1919). Examined in three dimensions, the bladder-like nature of the ectoplasmic extrusions is readily observable.

It was possible to resolve one controversy which occurs in the literature concerning the possible identity of the bladder amebocytes and the filiform amebocytes in asteroids. Théel (1919) and Kindred (1924) state these are merely phases of one another but cite no convincing evidence, and others question this conclusion. In observations on body fluids of several asteroids, the fresh sample showed a predominance of bladder amebocytes, but upon standing, the same preparation shows a predominance of filiform amebocytes. If the filiform amebocytes represent a pre-coagulation change, it should be possible to prevent this with an anticoagulant such as cysteine. Cysteine-treated coelomic fluid was found to contain only bladder amebocytes when examined at various time intervals in *Pisaster ochraceous* body fluid. This experiment was repeated eight times with the same results. In the control, samples of coelomic fluid were treated with sea water equal in volume to the sample of anticoagulant and upon standing, both phases were seen. Whether such transformation occurs in all echinoderm coelomic fluids in which such cells are found remains to be seen.

Some problems are presented by the present study of echinoderm coelomic fluid which may be of special interest to comparative and cellular physiologists. The function of the echinochrome-containing eleocytes and the various types of amebocytes still remains a challenge. The function of the vibratile corpuscles of the sea urchins, with the possibility that they represent parasites, is another example of an intriguing problem. The bladder amebocytes and the explosive amebocytes should serve as interesting material for a further study of ameboid movement. The mechanism of the transformation of bladder amebocytes to filiform amebocytes offers still another perplexing problem.

The data so far gathered do not permit evolutionary speculations concerning the origin and diversification of the different types of coelomocytes. However, it cannot escape mention that a greater diversity of cell types appears in the body fluid of the more highly specialized forms, such as the echinoids, than in the asteroids. A more complete survey of the coelomic corpuscles of other species of each class, especially of the classes studied sparingly at present, may yield information making possible more generalizations than can be made now.

SUMMARY

1. The cellular elements from the body fluid of 15 different species of echinoderms were studied by phase contrast microscopy. Thirteen types of corpuscular elements were identified and the distribution, properties, characteristics and, where possible, functions, were determined.

2. Some types of coelomocytes were observed in the species examined here which had not been previously described, *e.g.* the red corpuscles of the sand dollar and crinoid, and the lobular corpuscles of the crinoid. Some of the coelomocytes formerly described were also found in the species described. Among these are the controversial bladder amebocytes in which the presence of bladder has been questioned. Present studies verify the bladders as real structures easily seen in three dimensions. The bladder amebocyte undergoes a transformation into the filiform amebocyte which represents a pre-coagulation change.

3. A greater diversity of cell types was observed in the body fluid of the more highly specialized forms such as the echinoids than in the less specialized asteroids.

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THE ROLE OF THE BLOOD IN THE TRANSPORTATION OF STRONTIUM^{90}–YTTRIUM^{90} IN TELEOST FISH [1, 2]

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As the result of global fallout and the introduction of radioactive wastes from nuclear reactor plants into the oceans, marine organisms are being subjected to an environment which is potentially hazardous to themselves and to other members of the ecosystems involved. During the last few years, a study has been made in this laboratory of various aspects of the metabolism of radiostrontium by marine fish. These fish may pick up strontium directly from sea water, by way of the skin, gills, or by swallowing the water (Boroughs, Townsley and Hiatt, 1956). They may also take up this element from their food. In any event, the transportation of strontium within the fish, including its excretion, depends upon its transportation by the blood, except for the strontium which is unabsorbed from the digestive tract.

It is the purpose of this paper to report on certain aspects of the transportation of strontium^{90}-yttrium^{90} in teleost blood.

Materials and Methods

The species used in this experiment was *Tilapia mossambica,* a teleost fish. Individuals weighed between 50 and 110 grams each. They were kept in tanks supplied with running sea water.

Two concentrations of Oak Ridge Sr^{90}-Y^{90} were prepared by dilution with saline solution approximately isotonic with *Tilapia* blood. Those fish which were to be bled a day or more after injection were given 100μc of Sr^{90}, while the fish killed at shorter time intervals were given only 10 μc. In both instances the dose injected was 0.2 ml.

The injections were made, and blood was withdrawn with the fishes' opercula in water. Separate fish were used for each time interval studied instead of using a single fish for repetitive bleedings. All the fish were handled as gently and uniformly as possible, and their eyes were covered with the hand. We believe this procedure results in a minimum of trauma.

The Sr^{90}-Y^{90} dose was injected directly into the ventricle of the heart. At predetermined time intervals of 5, 15, 30, and 45 minutes and 1, 4, and 8 days, as much as possible of each fish's blood was withdrawn through the kidney sinus. A red blood cell count was made each time a fish was injected and again when blood was removed.

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[2] This work was supported in part by contract No. AT(04–3)–56 between the U. S. Atomic Energy Commission and the University of Hawaii.

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Immediately after removing the blood from the fish, triplicate 0.1-ml. samples were pipetted onto circles of one thickness of absorbent tissue on aluminum planchettes. Three-tenths-ml. aliquots of the remaining blood were centrifuged for 10 minutes at 2100 rpm in calibrated small bore hematocrit tubes in an International clinical centrifuge. The separated blood in one tube was used for measuring the radioactivity in the plasma and also that associated with the cells. From a second tube the plasma was removed without disturbing the packed cells. Five-hundredths ml. of these cells were washed by re-suspending them twice in fresh saline solutions. All the saline washings were pooled. In a third tube, the same volume of saline-washed cells was lysed with distilled water. The ghosts were washed with distilled water until no further radioactivity could be removed from them. The lysing solution containing the cell contents was added to the distilled water wash for measurement of the radioactivity of the cells exclusive of that bound to the stroma.

Separated organs and tissues were ashed and prepared for counting as previously described (Boroughs, Townsley and Hiatt, 1956). Radioactivity was measured with a thin window G-M tube using a commercial scaler. Counts were corrected for coincidence whenever necessary.

In order to get an approximation of mixing time, Sr^{85} was injected in the heart. Ten, 20, and 30 minutes later, blood was removed from the ventral aorta and from the kidney sinus, and 0.1-ml. samples were counted in a well scintillation counter with the aid of a single channel pulse height analyzer.

Results and Discussion

Preliminary experiments

Since very little is known about fish blood, we were at the outset faced with problems which were not pertinent to the main idea of this research. The first problem to be overcome was the bleeding, because apparently very few biologists have successfully removed blood directly from teleost fish (Prosser, personal communication). In general, fish have been bled by cutting the tail and allowing the blood to drip. Even more refined methods have involved the use of heparin, citrate, or other anticoagulants. We have found it difficult to withdraw unclotted blood from *Tilapia* if the fish had been kept out of water for even a short time. There is probably a dehydration of the blood in some species of fish as a result of asphyxiation (Hall, Gray and Lepkovsky, 1926). If *Tilapia* were stressed by prolonged chasing with a net, by rough handling or by repeated bleeding, removal of blood was difficult even though they were not taken from the water. The cell/plasma ratio increased as it did with asphyxiation.

We had previously observed red blood cell counts which varied between 1 and $4 \times 10^6/\text{mm}^3$ in this species of fish, and other workers (Young, 1949) have observed similar large variations with other teleost fishes. Table I is a summary of the rbc counts of the fish used in this experiment and shows that these variations are not intrinsic and that it is possible to remove fish blood that has a reasonably small fluctuation in the rbc count. This blood does not clot even on prolonged standing at room temperature.

The tremendous shift in the number of red blood cells observed in fish blood

TABLE I

Red blood cell count in Tilapia mossambica

Time interval between injection and killing	RBC/mm.^{3} of blood	
	Counted before dose injected	Counted before blood withdrawn
5 min.	1.444×10^6	1.627×10^6
5 min.	1.150×10^6	1.423×10^6
5 min.	1.375×10^6	1.400×10^6
15 min.	1.350×10^6	2.050×10^6
15 min.	1.209×10^6	1.374×10^6
30 min.	1.548×10^6	1.525×10^6
30 min.	1.175×10^6	1.400×10^6
45 min.	1.125×10^6	1.460×10^6
1 hr.	1.200×10^6	1.600×10^6
1 hr.	1.162×10^6	1.384×10^6
1 hr.	1.050×10^6	1.025×10^6
2 hr.	1.223×10^6	1.347×10^6
2 hr.	1.148×10^6	1.326×10^6
2 hr.	1.150×10^6	1.220×10^6
4 hr.	1.151×10^6	1.169×10^6
4 hr.	1.209×10^6	1.137×10^6
8 hr.	1.199×10^6	—
8 hr.	1.011×10^6	1.102×10^6
1 day	1.312×10^6	1.396×10^6
1 day	1.649×10^6	1.598×10^6
2 days	1.100×10^6	1.199×10^6
2 days	1.298×10^6	1.298×10^6
4 days	1.103×10^6	—
4 days	1.271×10^6	1.362×10^6
8 days	1.150×10^6	1.273×10^6
8 days	1.018×10^6	1.175×10^6

could mean that the plasma, or some portion of it, either leaves the circulatory system or is in effect removed by some pocketing device. The increase in red blood cells may also result from the introduction into the blood stream of cells previously sequestered in an organ or tissue. Studies on fish blood volume and mixing time using either classical techniques or radioisotopes would be of little value if the fish were stressed.

The circulation of fish blood is distinguished from that of higher animals in that oxygenated blood does not necessarily return to the heart. All the blood from the heart goes to the gills, but from the gills the blood may go to the head,

TABLE II

The mixing time of Tilapia blood

Blood source	Minutes elapsed	Counts/min.
Ventral aorta	10	249
Ventral aorta	20	79
Ventral aorta	30	51
Kidney	10	40
Kidney	20	45
Kidney	30	50

Dose: 8477 cpm in 0.2 ml. injected into ventricle of heart.

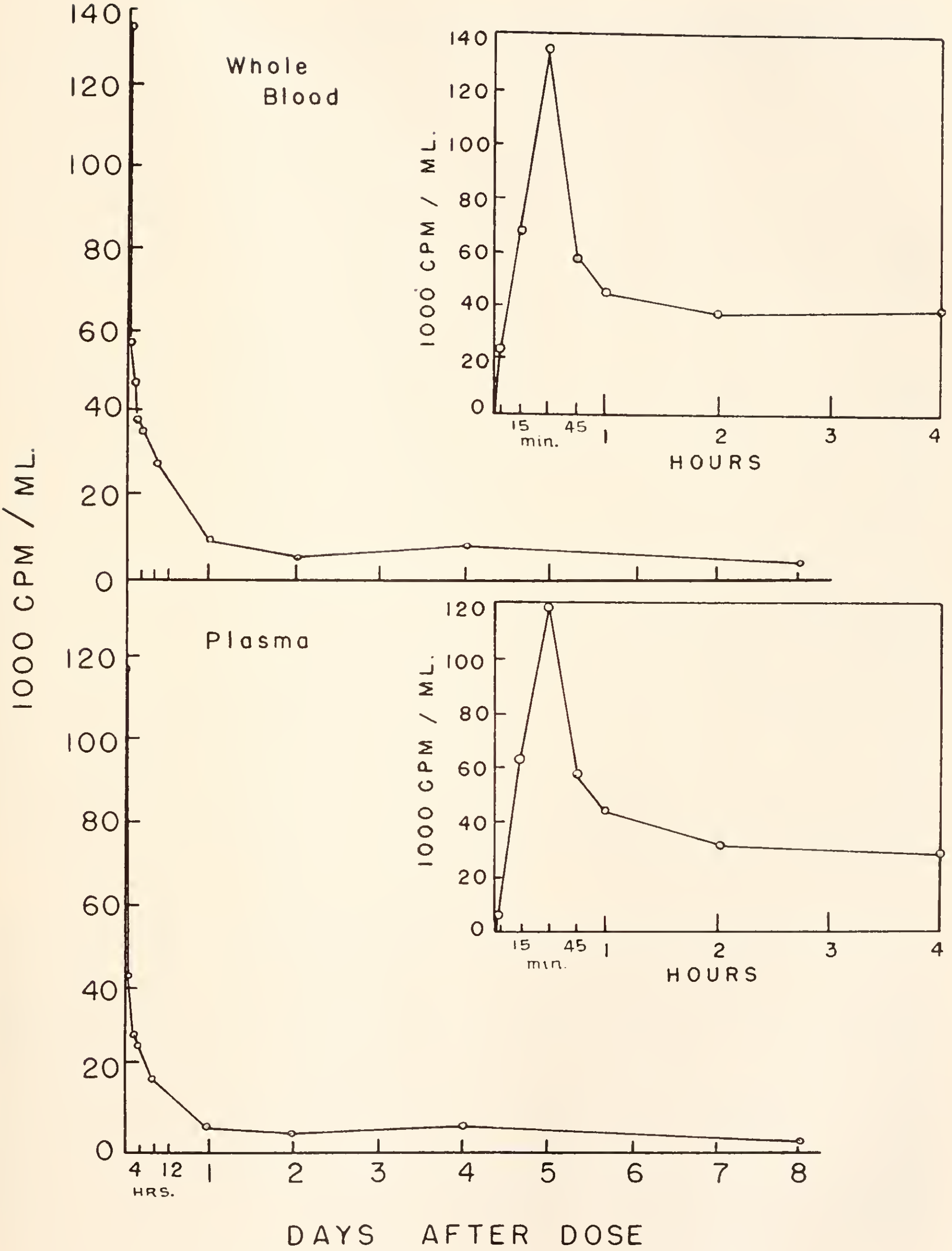

FIGURE 1. The disappearance of Sr^{90}–Y^{90} from the whole blood and plasma of *Tilapia mossambica*.

back to the heart, or to the remainder of the body. This means that mixing is a more complicated process in fish than it is in the higher animals.

The results of studying mixing time in a single fish are shown in Table II. It can be seen that the bulk of the Sr^{85} injected into the heart remained in the anterior portion of the fish, and that it required about 30 minutes for the blood from the ventral aorta and that from the kidney to reach the same level.

Since we lack precise information about blood volume, we have assumed that it is roughly proportional to body weight. We have done this not only on the basis of our own work, but because Martin (1950) has suggested a similar relationship for other teleost fishes.

Rate of disappearance of Sr^{90}-Y^{90} from the blood

Figure 1 shows the rate of disappearance of Sr^{90}-Y^{90} from whole blood and plasma. The numbers have been corrected for body weight. The activity is given in counts/min./ml. whole blood and cpm in the plasma present in 1 ml. of whole blood. Each point on the curve represents the average activity from at least two fish. It can be seen that practically all the radioactivity in the whole blood is carried in the plasma, and that the formed elements can be responsible for only a very small amount. The two curves are practically superimposable. The small inserts on this graph show the appearance of radioactivity during the first few hours, and the larger graph extends the curves to 8 days. Since all the radioactivity was injected into the heart at zero time, at first glance it may seem odd that the amount of radioactivity recoverable from the blood *increases* up to 30 minutes. However, Table II indicates that this apparent increase is a reflection of the mixing time. At least two processes are occurring during this time which make it extremely difficult to find out exactly how much radioactivity is in the blood system. First, the isotopes are being excreted as soon as they appear in the blood, at first principally by way of the gills. Second, radioactivity is rapidly accreted by the various organs and tissues, and thus the concentration is decreasing continuously. We would like to emphasize that it is the resultant of these processes that is being measured.

The radioactivity was very rapidly lost from the blood during the next 30 minutes, and after 24 hours, only between 0.8 and 1.6 per cent of the injected dose remained in the blood, assuming a blood volume of 2–4 per cent of the body weight. The shape of the curves shows that more than one rate process is involved in the disappearance of the radioactivity from the blood. It must be emphasized at this point that the above samples were counted at least three weeks after the fish was killed, so that we were observing the radioactivity in an equilibrium mixture of Sr^{90}-Y^{90}. $Strontium^{90}$ has a half-life of about 28 years and a maximum beta energy of 0.61 Mev. It decays to form radioactive Y^{90} which has a half-life of 2.54 days and a maximum beta energy of 2.18 Mev. Secular equilibrium exists when the Y^{90} decays as fast as it is formed, and the radioactivity of such a mixture is the sum of the radioactivity of the separate isotopes.

In an equilibrium mixture, therefore, no decay of radioactivity would be observable during this experiment unless the two isotopes were separated by either biological or physico-chemical processes. Such a fractionation can be detected by following the counting rate of a sample daily. No changes in this rate will be observed if no fractionation has occurred. If the rate increases, Y^{90} has been removed and is building up to its equilibrium value at which point it will level off. If the rate decreases, the bulk of the radioactivity must be due to the Y^{90} which is decaying, and the counts will decrease until a level is reached which is a function of the amount of Sr^{90} present.

The role of the blood fractions in the transport of Sr^{90}-Y^{90}

The increase in the counts/minute of the whole blood and plasma in Figure 2 is due to the build up of Y^{90}. There are two simple explanations for the loss of yttrium from the blood. One is that the yttrium was lost prior to its appearance in the blood initially, that is, adsorbed to the glassware used in making the dilutions and injections. The second explanation is that the yttrium was lost to various organs and tissues through which the blood passed. These explanations are not mutually exclusive and we believe that both processes occur.

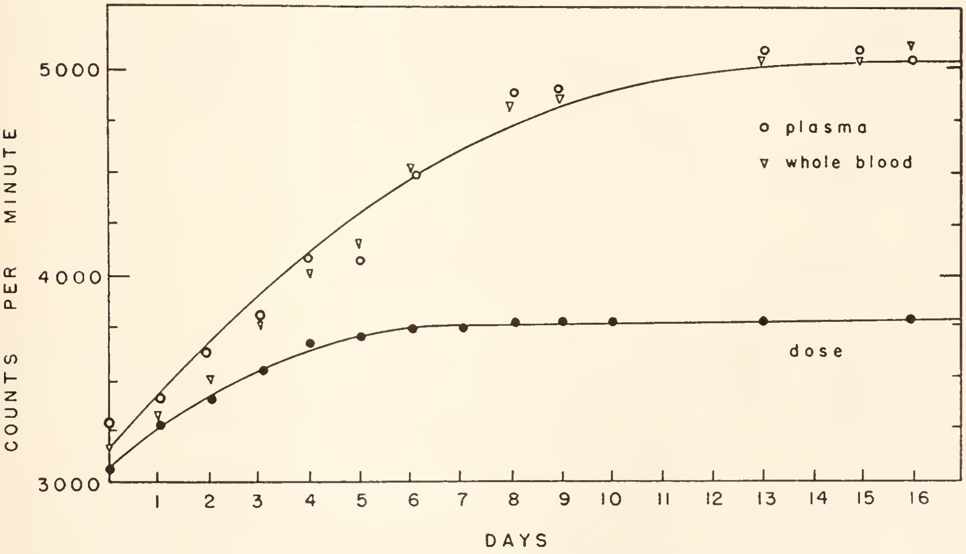

FIGURE 2. The increase with time of radioactivity in samples of whole blood, plasma, and the dose, indicating the build-up of Y^{90}.

In Figure 3, the curve labelled "dose" was obtained by counting planchettes prepared from the Sr^{90}-Y^{90} present in the syringe used for injections. It can be seen that over a period of time, the cpm increased, indicating that some Y^{90} was lost from the equilibrium mixture. This Y^{90} was lost to the glassware. The curve for whole blood and plasma, however, increased to a much higher value, indicating that additional Y^{90} had been removed after the dose was injected.

Figure 3 shows the rate of radioactive decay of the washed and unwashed cells, the saline washings, the washed ghosts, and the distilled water washings which include the cell contents. The decay of the unwashed cells suggests that both Sr^{90} and Y^{90} were associated with the cells. The decay of the washed cells, saline wash, and ghosts, however, suggests that the Sr^{90} is readily removable either from or through the cell wall. The activity remaining in the washed cells and ghosts indicates it to be Y^{90}, because the decay rates are very similar to the rate for pure Y^{90}. All these conclusions are in harmony with the findings of Thomas *et al.* (Thomas, Litovitz, Rubin and Geschickter, 1950), who showed that radiocalcium, metabolically similar to strontium, was carried in the plasma of rabbit blood.

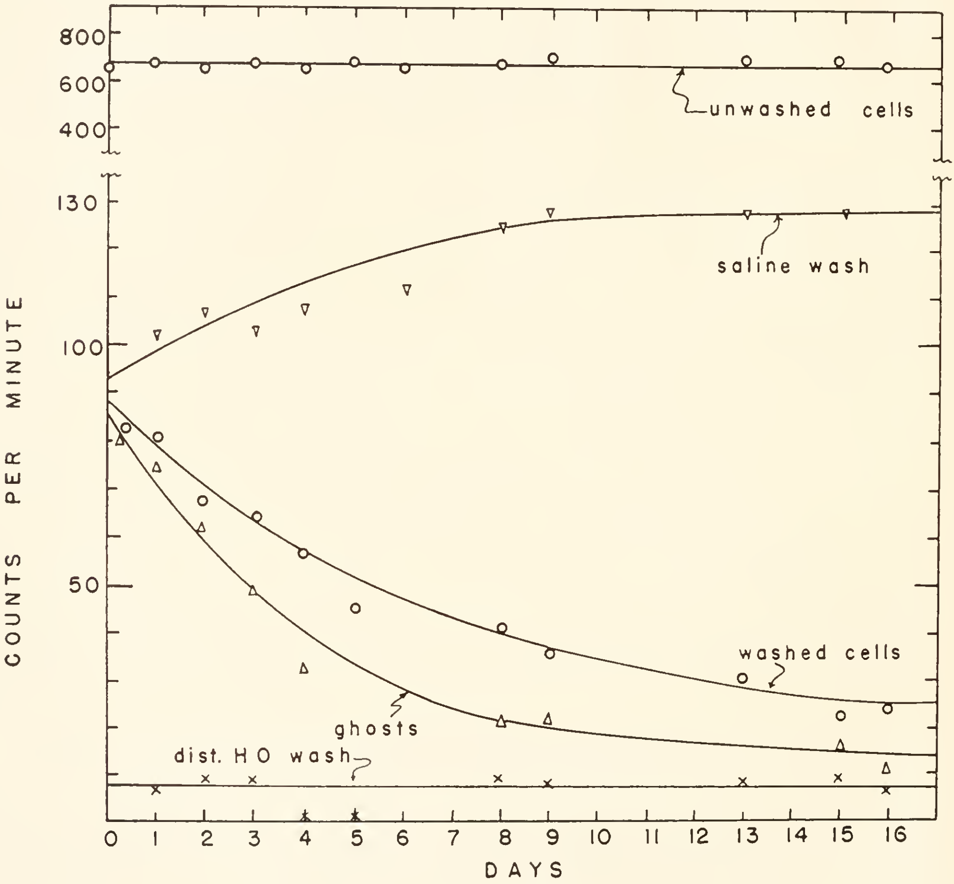

FIGURE 3. The radioactive decay of washed and unwashed cells, the saline and distilled water wash, and the cell ghosts. The decay of washed cells and ghosts indicates that they pick up Y^{90} rather than Sr^{90}.

Retention and distribution of Sr^{90}-Y^{90}

Figure 4 shows the retention by the fish of the injected Sr^{90}-Y^{90} as a function of time. The upper curve represents the entire fish, and the other curves represent, respectively, the bone, integument, gills, muscle, and visceral organs. Each point is the average of at least two fish, and the samples were counted at secular equilibrium. These results may be compared with those obtained previously by

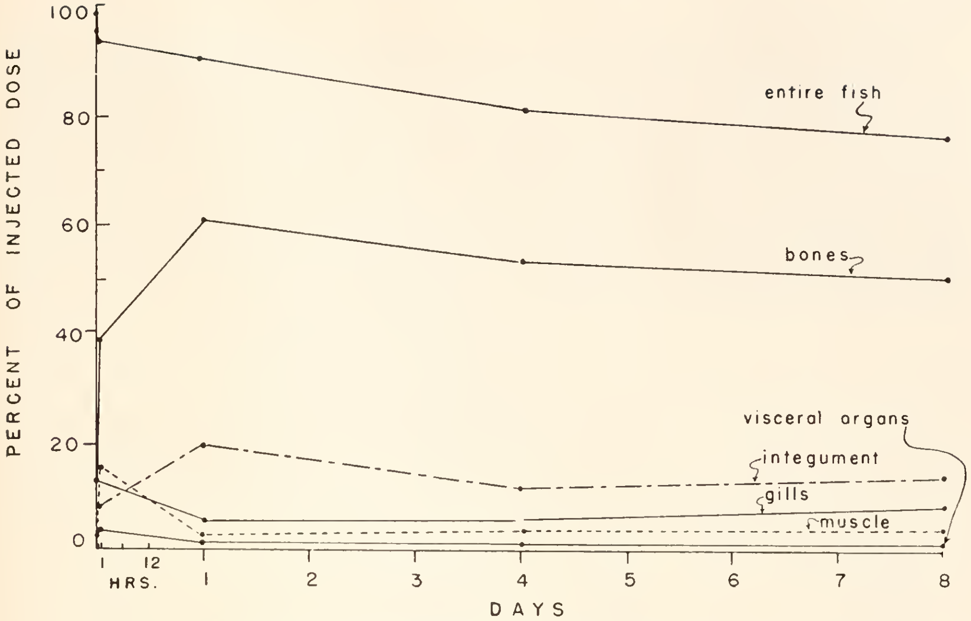

FIGURE 4. The internal distribution of Sr^{90}–Y^{90} injected in the heart of *Tilapia mossambica.*

Boroughs *et al.* on the retention of Sr^{89} by *Tilapia* following ingestion or intramuscular injection. In all instances, the rank order of the percentage of dose retained is the same as that in Figure 4, indicating that once the strontium is absorbed by the blood system, its internal distribution is the same.

Table III emphasizes the rapidity with which the radioactivity appears in the various tissues, including the bones. A small amount of vascular tissue and blood is dissected with the bones, but this additional radioactivity is obviously a very small percentage of the total in the bones. This appearance and retention in the bones cannot be due to accretion by growth, but must represent simply an exchange reaction.

TABLE III

Retention and internal distribution of Sr^{90}–Y^{90} after intracardial injection

Time interval	% injected dose remaining (samples at secular equilibrium)					
	Total of fish*	Bones	Integument	Gills	Muscle	Visceral organs
5 min.	98.2	13.2	13.4	13.4	3.1	3.4
15 min.	95.9					
30 min.	93.2	39.3	8.2	17.3	11.9	4.0
1 day	92.1	61.7	19.2	4.9	4.5	1.4
4 days	81.5	53.7	17.1	4.7	4.2	1.4
8 days	76.6	50.2	16.2	7.4	3.3	1.4

* Including blood.

Biological fractionation of Sr^{90}-Y^{90}

Three fish were injected with Sr^{90}-Y^{90} and killed five minutes, 30 minutes, and one day later. Since the amount of separation of the two isotopes by the glassware was unknown, it is not possible to draw a curve showing the rate of decay of the radioactivity in the various organs that would be a true measure of the decay due to the fractionation by the organs themselves. The planchettes were counted one day after the fish were killed, and this value was taken as a base line. They were then counted until secular equilibrium had been re-established. Table IV shows the percentage increase or decrease in radioactivity in the various organs with respect to the radioactivity present at one day.

Table IV

Fractionation of intracardially injected Sr^{90}–Y^{90} by organs and tissues of Tilapia mossambica

Organ or tissue sample	% Decrease of activity 1 day to secular equilibrium	Organ or tissue sample	% Increase of activity 1 day to secular equilibrium
Liver	53.1	Gills	73.8
Gall bladder	42.5	Stomach	22.3
Heart	38.6	Brain	18.3
Kidney	18.8	Muscle	16.3
Spleen	10.6	Intestine	10.4
Gonads	8.5	Eyes	6.9
		Urinary Bladder	5.6
Urine	28.5	Skin	5.0
Blood clots	26.8		
Scales	14.1		
Fat	11.4	Feces	2.1

It can be seen that the first two columns represent the organs which concentrated Y^{90} more than they did Sr^{90}, while the last two columns represent organs that favored the Sr. In general, the more vascular organs and tissues preferred yttrium.

Summary

1. Blood can be easily removed without clotting from the heart or kidney sinus of fishes if the fish are handled gently and their opercula are kept immersed.
2. Blood so removed has a uniform number of red blood cells/mm^3.
3. The mixing time of Sr^{90}-Y^{90} injected in the ventricle of *Tilapia mossambica*, a teleost fish, is approximately 30 minutes.
4. Sr^{90}-Y^{90} rapidly disappears from the blood. At 24 hours, only between 0.8 and 1.6 per cent of the injected dose remains in the blood.
5. The disappearance of radioactivity from the blood depends on more than a single process.
6. Almost all of the Sr^{90} in whole blood is carried by the plasma.
7. Very little Sr^{90} is found either in the cells or on the cell walls.
8. Yttrium^{90}, on the other hand, is present in the stroma.

9. The pattern of internal distribution of intravascularly injected Sr^{90}-Y^{90} is the same as that which was found for either intramuscular or oral administration in the same species.

10. Vascularized tissues have a greater avidity for Y^{90} than they have for Sr^{90}.

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DISPERSAL OF THE GELATINOUS COAT MATERIAL OF MELLITA QUINQUIESPERFORATA EGGS BY HOMOLOGOUS SPERM AND SPERM EXTRACTS[1]

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Live sperm or sperm extracts of a number of animal species have been found to possess the property of solubilizing or dispersing the secondary and tertiary envelopes normally surrounding the unfertilized eggs of these species, thus facilitating the approach of the sperm to the egg surface. Groups in which this phenomenon has been demonstrated include amphibians (Hibbard, 1928; Wintrebert, 1929 and 1933), mammals (see reviews by Duran-Reynals, 1942; Meyer, 1947; Meyer and Rapport, 1952), gastropods (Tyler, 1939 and 1948; von Medem, 1942), and bivalves (Berg, 1949). In addition, a number of workers have described the solubilization of the gelatinous coat material (fertilizin) of echinoid eggs by live sperm or sperm extracts. Hartmann *et al.* (1940) extracted the residue of methanol-precipitated seminal fluid of *Arbacia pustulosa* with sea water and reported that the resulting solution was capable of dispersing the gelatinous coat material of unfertilized Arbacia eggs. This extract was also capable of neutralizing the sperm agglutinating property of Arbacia fertilizin, and thus possessed antifertilizin activity. Monroy and Ruffo (1947) described an acid extract of sea urchin sperm which was reported as acting to dissolve the fertilizin of unfertilized eggs. Others have described a decreased viscosity of fertilizin solutions in the presence of live sperm or sperm extracts (Lundblad and Monroy, 1950; Vasseur, 1951; Monroy and Tosi, 1952; Monroy *et al.*, 1954). It has been emphasized (Tyler and O'Melveny, 1941; Krauss, 1950; Monroy and Tosi, 1952; Monroy *et al.*, 1954) that apparent dispersal of the gelatinous coat of unfertilized eggs by sperm or sperm extracts, as well as the decrease in viscosity observed when live sperm or extracts are added to fertilizin solutions, can be accounted for by precipitation of fertilizin by antifertilizin present in the extracts or on the surface of the live sperm. Therefore, any investigation of supposed lytic or dispersing agents from sperm must include experiments which demonstrate that the activity of the agent is separable from the activity of antifertilizin. Ishida (1954) has presented evidence that a fertilizin-dissolving factor is released at fertilization from the sperm of *Hemicentrotus pulcherrimus*. Treatment of the sperm with fertilizin, which rendered the sperm non-fertilizing, did not prevent the solution of the fertilizin coat of the eggs by these sperm. This latter observation tends to eliminate antifertilizin as the agent responsible for removing the fertilizin from the eggs. However, though the sperm concentration employed in the experiments was not stated, sperm carbon dioxide might have been responsible for the solubilizing action of the sperm. Heated (100° C. for one minute) sperm failed to cause dispersal of the fertilizin coat. This failure of heated sperm to cause dispersal of the gelatinous coat has been ascribed, by Ishida, to the denaturation of a dispersing agent on the sperm. Alternatively, the denaturation of respiratory enzymes, resulting in loss of motility and decreased carbon dioxide production, could account for the failure of heated sperm to solubilize the fertilizin of the eggs.

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[1] This investigation was supported in part by a research grant (RG 4659) from the National Institutes of Health of the Public Health Service.

The present report bears evidence that a factor, which is distinct from antifertilizin and which is capable of causing the dispersal of the gelatinous coat of unfertilized eggs, is present in sperm extracts and on the surface of live sperm and of the sand dollar, *Mellita quinquiesperforata.*

MATERIALS AND METHODS

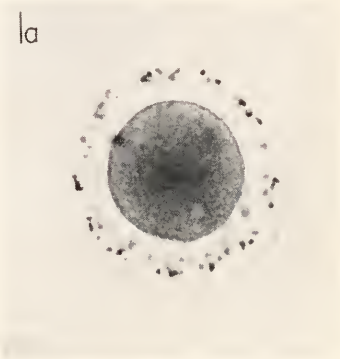
Animals were collected by dredging on the shallow banks surrounding the University of Florida Marine Laboratory at Sea Horse Key. The animals were transported to Gainesville and kept in the laboratory at 12° C. in sea water supplied with a continuous flow of washed, compressed air. Under these conditions the sand dollars remained alive for approximately two weeks. Eggs and sperm were obtained by injecting the animals with an isotonic KCl solution (Tyler, 1949).

For use in experiments in which live sperm were employed, the "dry" sperm were diluted to a concentration of 5% with filtered sea water. Where separation of sperm from the surrounding fluid was desired, the suspensions were centrifuged at 2900 × gravity in a Servall SS-1 centrifuge for 10 minutes. The supernatant fluids were collected and tested for dispersing activity on the gelatinous coat of fresh unfertilized eggs. The sedimented sperm were suspended in the original volume of fresh sea water and tested for their ability to disperse the gelatinous coat of the eggs.

Sperm extracts were prepared from washed sperm in the following manner. Two volumes of sea water were added to the sperm following centrifugation and removal of seminal fluid, and the resulting 30% suspension was frozen at − 20° C. for 2 to 12 hours. The frozen suspension was then homogenized in an ice bath, using a Potter homogenizer with a motor-driven pestle. After homogenization, the suspension was centrifuged at 11,000 × gravity for 15 minutes in a Servall SS-1 centrifuge. This procedure yielded a gray precipitate, which was discarded, and an opalescent supernatant fluid, which was used as the final sperm extract preparation.

In assaying for the dispersing action of sperm and sperm extracts on the gelatinous coat of the eggs, advantage was taken of the presence of echinochrome granules in the gelatinous coat. As can be seen in Figure 1a, where the outer boundary of the coat has been outlined with antifertilizin, the granules normally have a rather regular position in the gelatinous coat. The locus of this position could be described as a spherical shell lying midway between the outer surface of the gelatinous coat and the surface of the egg. In practice, a small number of freshly shed eggs were transferred with a pipette from the vessel in which they had been allowed to settle, to fresh sea water, and used in the various tests. Dispersal of the egg coat could be followed by noting the length of time required for the echinochrome granules to fall to the bottom of the culture dish, due to the dispersal of the gelatinous matrix in which they were embedded. The time at which the granules were released represented the time at which approximately half the gelatinous coat had been dispersed.

and was taken as the end-point of the reaction. Naturally, every effort was made to insure that the eggs used in the experiments possessed comparable amounts of gelatinous coat material outside the layer of granules. In practice, this was not particularly troublesome since the egg coat of Mellita eggs is quite rigid and not readily soluble in sea water, and since handling of the eggs was restricted to a single transfer to fresh sea water following shedding.



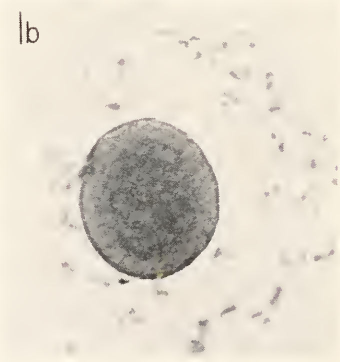


FIGURE 1a. Unfertilized egg of Mellita treated with heated (70° C. for 10 minutes) sperm extract. Final magnification: 175 ×.

FIGURE 1b. Unfertilized egg of Mellita treated with heated sperm extract for 30 minutes, followed by treatment with unheated extract for 15 minutes. Final magnification: 175 ×.

RESULTS

When one drop of 5% sperm suspension was added to one drop of egg suspension (containing about 200 eggs), the gelatinous coat was dispersed in approximately 10 minutes. Elevation of the fertilization membrane did not result in the dispersal of the gelatinous coat of control eggs which were washed and removed to sea water following exposure to the sperm suspension. When the above sperm suspension was centrifuged at 2900 × gravity for 10 minutes, and the supernatant fluid decanted and tested, it was found to be inactive (no dispersal of the gelatinous coat occurred even after 12 hours of exposure to the sperm supernatant). The sedimented sperm, on the other hand, remained able to disperse the gelatinous coat material after being diluted to the original concentration with sea water.

Acidification of a 5% sperm suspension to pH 4, followed by return to pH 8 after 2–4 minutes, with subsequent centrifugation at 2900 × gravity to recover sperm and supernatant fluid, resulted in the loss of the capacity of the sperm to disperse the fertilizin coat. The sperm, in most instances, remained motile following the treatment. The supernatant fluid under these conditions occasionally showed slight antifertilizin activity, as evidenced by the formation of a slight precipitation membrane on the gelatinous coat, but remained inactive with respect to the dispersal of the gelatinous coat. The fertilizing capacity of such acid-treated sperm was reduced, perhaps due to the loss of antifertilizin from the sperm surface (Tyler and O'Melveny, 1941), or to the loss of the ability to disperse the gelatinous coat, or both.

Sperm extracts, prepared as described in the previous section, were also capable of dispersing the gelatinous coat of unfertilized eggs. The final extracts, prepared from frozen-thawed sperm, had a pH varying between 6.9 and 7.1, depending on the particular preparation. Extracts at this pH range were capable of dispersing the gelatinous coat in 30 minutes at 25° C. Control eggs in sea water at pH 6.9 showed no release of echinochrome granules for 6 hours or more. Exposure of active sperm extracts to pH 4 for 3–5 minutes, followed by return to the original pH, inactivated the dispersing factor. The optimum pH for dispersing activity appeared to be 8, since dispersal occurred in approximately 10 minutes at this pH. Alkalinization to pH 9, followed by return to pH 8 after 3–5 minutes, partially inactivated the dispersing factor, dispersal occurring after 30 minutes in these preparations. Heating to 70° C. for two minutes in a water bath completely inactivated the dispersing factor. The heated extracts possessed definite antifertilizin activity (Table I). The heat stability of antifertilizin from sperm of sea urchins (Frank, 1939), and the key-hole limpet (Tyler, 1939) has been previously described. Weak antifertilizin activity was also evident in untreated extracts at pH 8. A faint precipitation membrane appeared on the surface of the fertilizin coat about two

TABLE I

The effect of temperature and pH on dispersing activity of Mellita sperm extract. Activity of extracts assayed at 25° C.

Date of preparation of extract	Exposed to pH	Tested at pH	Dispersal time	Antifertilizin activity
Dec. 13	7	7	30′	±
Dec. 19	7	7	30′	±
Dec. 5	4	8	—	±
Jan. 17	4	8	—	±
Dec. 5	9	8	30′	±
Jan. 3	9	8	30′	±
Jan. 17	8	8	15′	±
Dec. 5	8	8	15′	±
Jan. 3	8	8	10′	±
Dec. 5*	8	8	—	+++
Dec. 19*	8	8	—	+++
Dec. 19*	8	8	—	+++

* Indicates heated extract (70° C. for two minutes).
— Indicates no dispersing activity evident.

minutes after the addition of extract, but disappeared after 5 minutes under the influence of the dispersing factor. This point was further illustrated by experiments with heated extracts possessing stronger antifertilizin activity. The heated extracts (70° C. for 5–10 minutes) formed definite precipitation membranes on the fertilizin coat after two minutes (Fig. 1a). The precipitation membrane so formed did not contract to the egg surface, but remained in the position in which it was originally formed for as long as 24 hours (with no dispersal of the egg coat). Addition of unheated sperm extract at pH 8 caused the disappearance of the precipitation membrane, and, after 10–15 minutes, the dispersal of the gelatinous coat (Fig. 1b).

Experiments involving the addition of extract to eggs up to five minutes prior to the addition of sperm showed that fertilization is not enhanced by this treatment. The fertilization membrane was elevated within three minutes regardless of the presence or absence of the extract. The sperm are apparently supplied with enough dispersing factor to make their way through the gelatinous coat material. Moreover, the gelatinous coat is not dispersed by the dilute suspensions usually employed for insemination. In this respect, the situation parallels that of hyaluronidase of mammalian sperm, in that hyaluronidase added to inseminates does not enhance fertilization (Chang, 1947; Leonard *et al.*, 1947).

The Mellita sperm extracts were not tested on eggs of other species, and the degree of specificity of the dispersing agent is therefore not known at this time. An extract of frozen *Arbacia punctulata* sperm (5% suspension) failed to cause the release of the echinochrome granules of Mellita eggs, but showed strong antifertilizin activity (the precipitation membrane with enclosed granules contracted to the egg surface in 5 minutes). Addition of extract of Mellita sperm, at pH 8.0, caused the gradual disappearance of the precipitation membrane formed by the Arbacia antifertilizin, and release of the echinochrome granules following dispersal of the fertilizin coat. In this connection, it is of interest that a fresh suspension of Mellita sperm was capable of fertilizing eggs treated with Arbacia antifertilizin, in the presence or absence of extract of Mellita sperm, indicating the ability of live sperm to penetrate precipitation membranes.

Further experiments were performed in the hope of discovering the means by which the Mellita sperm extract accomplished the dispersal of the gelatinous coat. A fertilizin solution, prepared by acid (pH 4) treatment of Mellita eggs, with a sperm-agglutination titer of 1 : 1000, was gently shaken with an equal volume of sperm extract at pH 8 for 60 minutes at 25° C. No decrease in titer of the fertilizin solution was evident at the end of the experiment. The sperm extract used in this experiment had been previously shown to disperse the gelatinous coat in 10 minutes, and showed no sperm-agglutinating property. The experiment indicated that no degradation of the fertilizin molecule resulting in loss of agglutinating activity occurred in the presence of the extract. Most probably, the dispersal of the gelatinous coat by sperm extract is accomplished by depolymerization of the gelatinous coat material, and not by splitting of individual fertilizin molecules.

Discussion

Since the dispersing factor is heat-labile under conditions where antifertilizin is stable, it seems reasonable to consider them to be separate substances. This conclusion is supported by the failure of strong antifertilizin solutions to cause dispersal of the gelatinous coat, even though a precipitation membrane forms and, in some instances, contracts to the surface. One can distinguish, therefore, between the dispersal of the gelatinous coat and its precipitation. Further, acid treatment of sperm or sperm extracts, a procedure not infrequently used for antifertilizin extraction from whole sperm (Tyler and O'Melveny, 1941), results in the inactivation of the dispersing factor, again indicating that the dispersing factor and antifertilizin are separate substances. Since the respiration of such acid-treated sperm is most probably normal (Tyler and O'Melveny, 1941), carbon dioxide is probably not involved in the dispersal of the gelatinous coat. The observed temperature and pH sensitivity of the dispersing factor suggest that it is protein in nature, possibly an enzyme.

The dispersing factor of Mellita sperm apparently does not act on fertilizin in solution, but only serves to liquify or disperse the gelatinous coat. If the fertilizin, in the gel state, is bound by cross linkages involving the area of the molecule capable of combining with antifertilizin, as Tyler (1948) suggests, the dispersing factor may operate by breaking such cross linkages, thereby releasing fertilizin from the gel. Further, the dissolution of fertilizin-antifertilizin precipitation membranes by extracts containing the dispersing factor may be due to the breaking of linkages at the fertilizin-antifertilizin combining site. Further experiments are necessary before the relationship of the dispersing factor to the fertilizin-antifertilizin reaction can be stated with certainty.

SUMMARY

1. A factor causing the dispersal of the gelatinous coat of Mellita eggs was shown to be present on the surface of Mellita sperm and in frozen-thawed extracts of sperm suspensions.

2. The factor was separable from antifertilizin on the basis of temperature and pH sensitivity.

3. The factor did not degrade fertilizin in solution, but released this substance from the gel surrounding the egg.

4. Active extracts were capable of dissolving fertilizin-antifertilizin precipitation membranes, formed on the surface of the fertilizin coat of unfertilized eggs in the presence of Arbacia or Mellita antifertilizin.

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AN EXOGENOUS REFERENCE-CLOCK FOR PERSISTENT, TEMPERATURE-INDEPENDENT, LABILE, BIOLOGICAL RHYTHMS[1, 2]

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The phenomenon of persistent rhythmicity of one or more of their vital processes is widespread among animals and plants. By persistent rhythmicity is meant that the rhythm still continues when conditions are held constant with respect to all factors generally conceded to influence the organisms.

Reviews of this subject have included those by Bünning (1936, 1956a, 1956b), Jores (1937), Kalmus (1938), Welsh (1938), Park (1940), Kleitman (1949), Calhoun (1944, 1945–46), Korringa (1947), Webb (1950), Caspers (1951), Cloudsley-Thompson (1953), Bruce and Pittendrigh (1957), and Brown (1957d, 1958). The broad distribution of such rhythmicity is suggestive of an hypothesis that all living things have potentially the means of persistent rhythmicity provided it has a period close to that of one of the natural geophysical rhythms. The organismic rhythms usually are essentially temperature-independent in their frequencies, whether the periods are solar-daily, lunar or annual.

Most of the observed rhythms are clearly endogenous, and are labilely adaptable in form and phase relationships to the needs of the organism. Much has been learned, particularly in recent years, as to the properties, including modifiability, of this endogenous rhythmicity. The fundamental problem, however, that of the timing mechanism of the rhythmic periods, has largely eluded any eminently reasonable hypotheses in terms of cell physiology or biochemistry. The problem was already a difficult one when only solar-daily cyclicity was under consideration, but especially in recent years it has been found that one and the same organism may simultaneously possess overt daily and lunar tidal cycles of two bodily processes. Further, the possession of persistent lunar monthly (Brown, Bennett and Webb, 1958) and even annual cycles (Bünning and Müssle, 1951; Bünning and Bauer, 1952; Brown, 1957c) in constant conditions has emphasized the magnitude and complexity of this basic problem.

Added to the property, temperature-independence, in indicating the unconventional character of the rhythm-timing mechanisms, are the repeated demonstrations of the immunity of the frequency-determining mechanism to most metabolic poisons.

Recently, evidence has been rapidly accumulating pointing to the possession by living organisms of basic metabolic cycles of the natural geophysical frequencies,

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[1] This study was aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, NONR-122803.

[2] The author wishes to express his appreciation to the several students who worked many hours in assisting to obtain and process the data used here. Thanks are especially due to Messrs. W. D. Korte, who handled the carrot experiments, E. F. Lutsch, F. H. Barnwell, E. J. Macey, H. Gibson, Jr., and Misses J. Strunk and B. Oetting.

produced in the organism by an external cyclic stimulus still operative in so-called laboratory constant conditions (Brown, 1957; Brown, Shriner and Webb, 1957; and Brown, Webb and Macey, 1957). These cycles are not phase- or form-labile. The problem of a common explanation for persistent rhythmicities of all the well-known natural frequencies including the year becomes at once more susceptible to reasonable working hypotheses as to their mechanism when it is firmly established that protoplasm in "constant conditions" is, fundamentally, exogenously rhythmic.

For the study to be reported here, the potato and carrot were selected as organisms neither of which appears to possess any obscuring, labile, endogenous rhythms. It was considered that such organisms would reveal most readily any extant basic protoplasmic cyclicities and also permit easier analysis of any mechanisms they involved.

On the basis of this hypothesis, of an exogenous reference clock providing the timing of cyclic periods, the often-described endogenous rhythms would be considered a consequence of the evolution by the organism of adaptive labile cyclic changes, utilizing the basic exogenous cycle-timing mechanism. The endogenous mechanisms could be inherited. The only inherited aspect of the exogenous cyclicity would be the fundamental protoplasmic responsive systems which are involved.

Materials and Methods

The potatoes, *Solanum tuberosum,* were of the Idaho variety and were purchased from local grocery stores. The carrots, *Daucus carota,* were similarly purchased from local stores. Using a cork-borer, small cylinders, 2.2 cm. in diameter and about 1.5 cm. tall, were prepared from the potatoes in such a manner that each carried an eye on the center of its upper surface. These were permitted to heal their cut surfaces before being set, in shallow water, in respirometer vessels where the same individual organisms were retained up to three or more months. These always gave rise to sprouts and usually also to a root system, and in some instances even developed new tubers up to a centimeter or more in diameter during their sojourn in the respirometers. For the carrots, short cylindrical sections, about the size of the potato-cylinders, were cut and allowed to heal over before being placed in respirometers.

The respirometers have been described earlier. These were originally designed by Brown (1954) and later modified (Brown, 1957a) to permit maintenance of constant pressure.

Five independent barostat-respirometer ensembles, each with 4 respirometers recording as a unit, were in nearly continuous operation during the period of study, Feb. 1, 1956 through Feb. 28, 1958. The potatoes in the respirometers were in constant illumination (estimated at 0.05 ft. c. at the site of the plants) from incandescent lamps supplied by a voltage-regulated line. The temperature, 20° C., was maintained constant by the respirometers being immersed in a large non-stirred, copper water-bath (the barostat) deeply immersed in an outer, stirred, steel (55-gal. drum) water bath, with the latter cycling with a few-minute period within a ± 0.05° C. range. The pressure was kept constant, 28.5 in. Hg, through hermetically sealing the respirometer-recorder-containing barostat and then aspirating the system to this level. Oxygen and CO_2 tensions were maintained essentially constant through use of the principle of continuous O_2-replacement together with CO_2 absorbents, and there were clearly no regularly cyclic fluctuations in these substances. Also, the sealed, water-included systems allowed for no changes in humidity.

With a single exception (12 days) the copper tanks, or barostats, remained sealed for periods ranging from 2 to 8 days, with an average of 4.46 days. At these intervals the organisms were exposed for 15–20 minutes to laboratory conditions which were relatively constant over the year. No work was done within 15 feet of outside windows; the laboratory fluorescent illumination at table top was about 45 ft. c. (The carrot study was carried out wholly in a dark-room without any natural illumination.) The room temperature was relatively constant, about 75° F., except for slightly higher values during the summer months. The barostats were opened at various hours of the day from 8 AM to 10 PM. Excluding those days the respirometers were opened to renew the O_2 and the CO_2 absorbent, a total of 2485 uninterrupted calendar days of data were obtained.

The recording systems of the respirometers possessed two points of slight mechanical frictional resistance, a) a two-point pivotal, spring-scale bearing, and b) the point of contact of the ink-writing pen with the slowly moving paper. These resulted in random, spurious apparent intra-hour fluctuations in rate of O_2-consumption. Since the principle of operation of the recorder was one with which the hourly values of O_2-consumption were obtained by calculating the differences between consecutive hourly markers on a continuing trend-line denoting cumulative O_2-consumption, these spurious fluctuations in apparent rate could, and undoubtedly did, produce larger hour-to-hour differences than bore any significance. Hence, time units of less than three hours (three-hour "moving means") were never used in determining the mean rates centered on any given hour. By this means the random mechanically induced error was reduced to about one-third its single-hour influence. For most of the study reported here, a weighted (1:2:3:3:3:2:1) seven-hour "moving mean" was used. This reduced by essentially 90% the random fluctuations while retaining all the precision of measurement of average, actual, O_2-consumption for this longer interval, as modified by its weighted character. The shorter period, three-hour, means were found necessary, however, to expose the relationship between day-by-day 6 AM deviations in O_2-consumption from daily linear trends and the concurrent day-by-day mean rates of barometric pressure change for the 2–6 AM interval. Although some clearly significant short-period fluctuations were obscured, therefore, by the seven-hour weighted "moving means," these were considered superior to the shorter periods for the accurate description of the general characters of the longer-period, daily and annual cycles to be described herein.

The records for the five completely independent, respirometer-recording systems were first dealt with individually and three-hour and weighted seven-hour "moving means" were prepared month by month for the period of study. From the latter values were calculated the mean daily rates of O_2-consumption and the data were then converted into hourly deviations from the solar daily means. The number of uninterrupted days of data from the 24 months of study ranged from 93 to 129 each month. The hourly deviations for all the respirometers operating were averaged for each calendar day, and these average daily cycles then converted to hourly deviations from a 1 AM to 12 midnight linear trend-line. This will be referred to as the deviations from linear daily trend. From these data the forms of the mean daily cycles for each month were obtained. The slope of this linear trend-line itself shows apparent monthly and annual periodisms which have been treated elsewhere (Brown, 1957c; Brown, Bennett and Webb, 1958). The trend involved a mean daily increase during the two-year study of 6.7%, and included, as a large

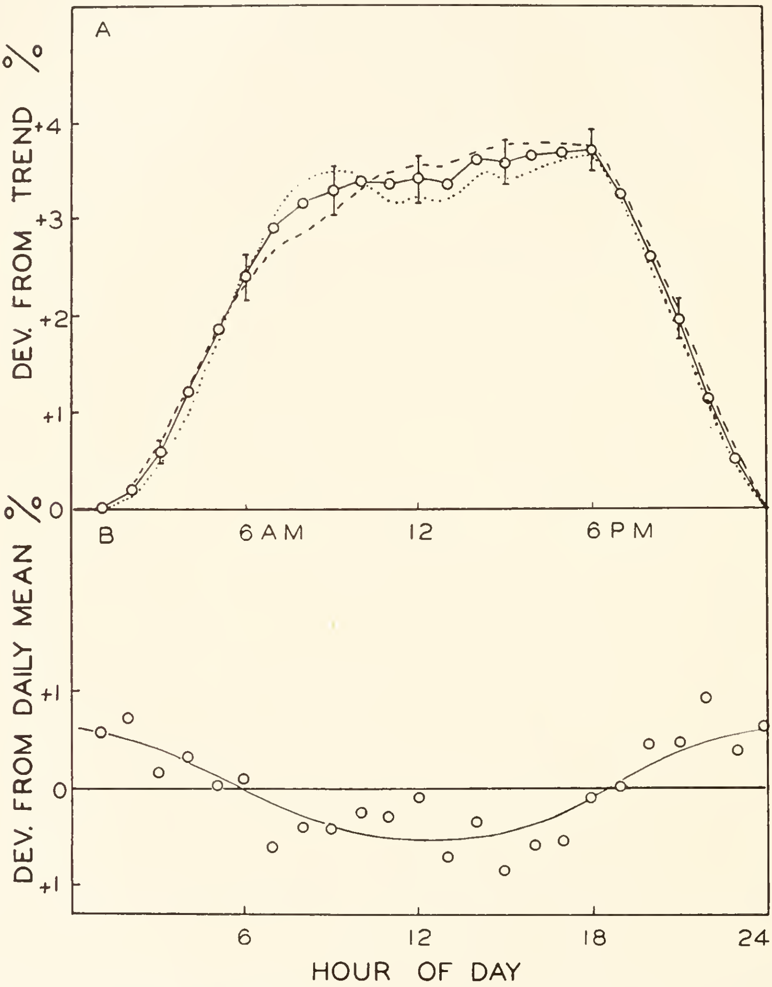

FIGURE 1. A. The mean solar-day cycle of O_2-consumption in the potato (solid line) with standard errors for selected hours. This is expressed as % deviations from linear daily trend. The dashed curve is the cycle for the first year of study, the dotted curve, for the second. B. The mean apparent sidereal-day cycle of the potato for the two-year period of study.

component, the apparent smoothly gradual recovery over a 3- to 5-day period, from the inhibitory influence of the room-illumination intensity. The mechanical recording system, itself, departed from linearity over its total range by 10%, departing in such a direction that there would be expected on this basis an average of about 2% increase per day.

An entirely independent and parallel study was made of O_2-consumption of the sections of the carrots, for the 8-month period Oct. 1, 1956 through May 31, 1957. Two respirometer-barostat ensembles were employed for the first three months, and four for the remaining five months. These were maintained in darkness in a photographic darkroom about 60 feet away from the place of the potato study, but similarly on the ground floor of Cresap Biological Laboratory, a three-story steel and mortar building. The respirometers were maintained and the data processed by a person not involved until the termination of the carrot study in the paralleling and continuing potato study.

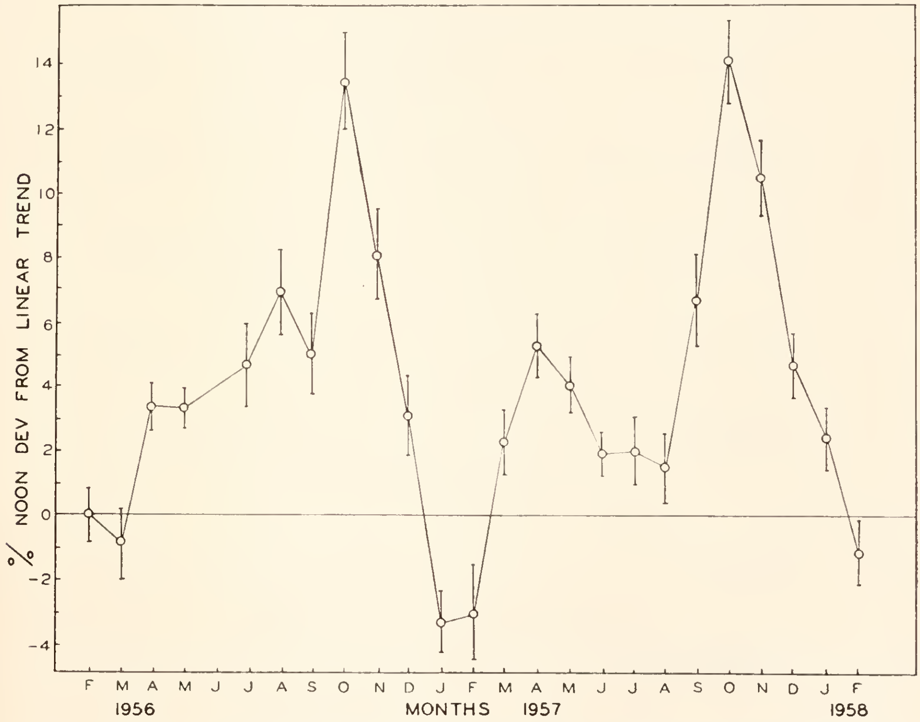

FIGURE 2. The relationship between average % noon deviation in O_2-consumption in the potato from linear daily trend and month of year during the 25-month study. Standard errors of means are shown.

RESULTS

During the period, Feb. 1, 1956 through Feb. 28, 1958, in the study of the potato, the only days omitted were May 25, the month of June and the first three days of July, 1956, and October 4, 1957.

The form of the mean daily deviation from trend, expressed as percentage of the daily mean rate, is shown, with the standard errors of arbitrarily selected mean values, in the solid-line curve of Figure 1, A. The errors of the other values are quite comparable in size. Superimposed on this are the mean cycles for each of the two years separately: Feb. 1, 1956 through Jan. 31, 1957 (the dashed curve) and

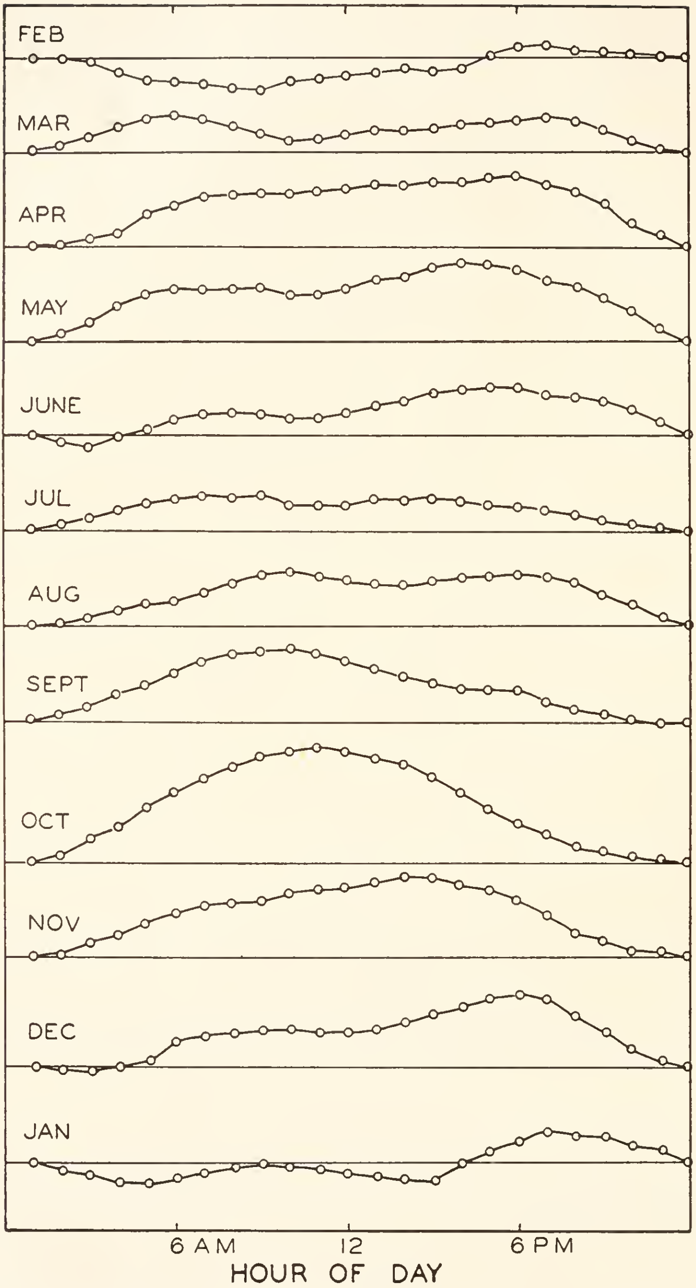

FIGURE 3. The forms of the average daily cycles for each month of the year obtained in the two-year study. The ordinate values are deviations in comparable arbitrary units.

Feb. 1, 1957 through Jan. 31, 1958 (the dotted curve). The average amplitude of the daily cycle was clearly quite reproducible for the two years at about 3.7%. There was also clear suggestion, in the skewed cycle form, of a bimodality with morning and afternoon maxima, a condition more conspicuous for the second than for the first year of study. The mean sidereal-day cycle (23 hours, 56.07 minutes) for the two-year period is shown in Figure 1, B. This was obtained by displacing

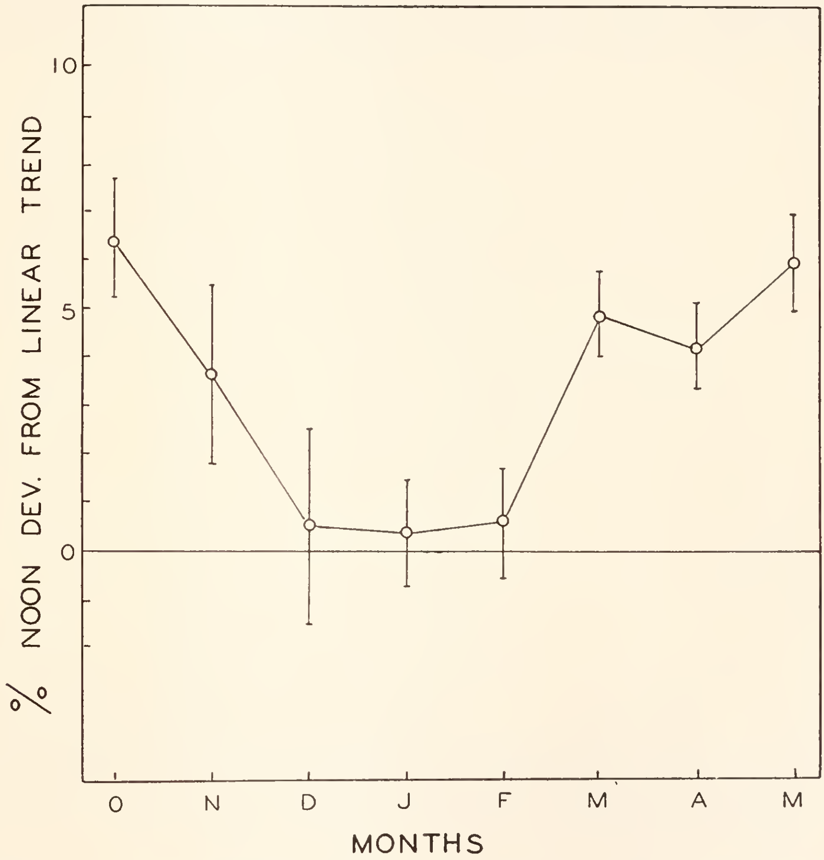

FIGURE 4. The relationship between the noon deviations in O_2-consumption from linear daily trend and calendar month in the *carrot* during an 8-month study. The standard errors are depicted.

the consecutive mean monthly solar-day cycles each by two hours to the right during the two-year period, to bring into reasonably close synchrony (± 1 hour) the hours of the sidereal day. The numbered hours are fixed by the solar-day hours of the first month, February, 1956. This process also randomizes daily trend.

The form and amplitude of the solar daily cycle showed differences from month to month which revealed that it was undergoing a modulation of an annual frequency. This was quite evident when one used, for example, the parameter of average monthly noon deviation, in percentage, from 1 AM to midnight daily linear trend. The deviations, month-by-month, for the period Feb. 1, 1956 through Feb. 28, 1958, together with their standard errors, are depicted in Figure 2. These indicate minimum annual values, involving often even apparent cycle inversion, during the coldest months of the year and a major maximum in the month of October. A lesser, or incipient, maximum occurred in April–May. The maximum range is seen to extend from − 3.4% to + 14.2%.

An annual cycle in over-all form of the mean daily cycles for the months of the year is evident in Figure 3, where twelve average cycles, the means for two years, have been plotted in terms of average deviation in arbitrary units from linear-trend.

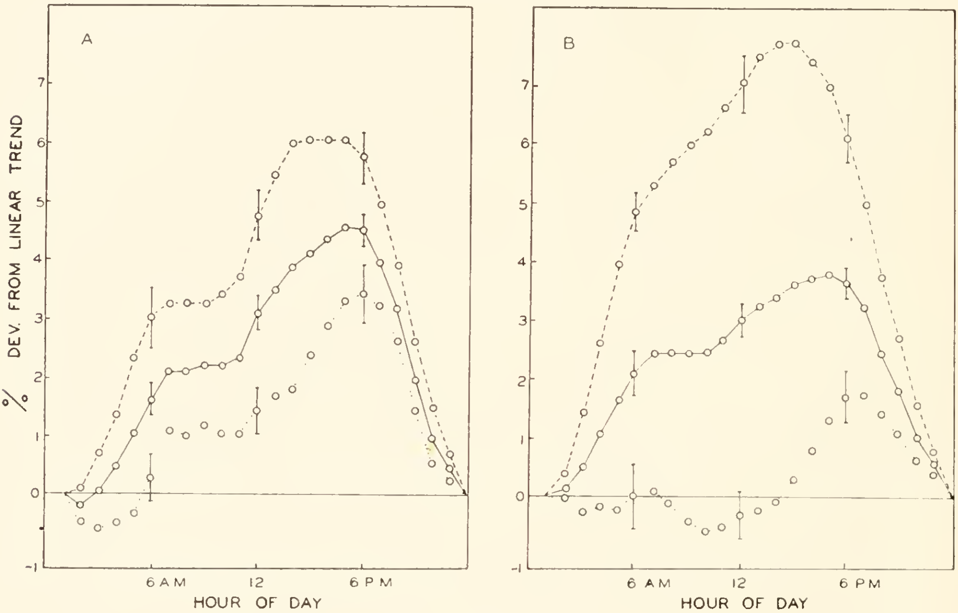

FIGURE 5. A comparison, for the same 8-month period of study, of the forms of the mean daily cycles for carrots (A) and potatoes (B). Solid curves show the mean cycles for the whole 8-month period. The dashed curves show the average cycles for October, November, April, and May. The dotted curves show the average cycles for December, January, February, and March. Standard errors of selected times of day are shown.

Although these data are not expressed as percentage deviations, they do illustrate the gradually-changing form of the cycles from unimodality with essential inversion in February, but with a 7 PM maximum, through a period of bimodality with the two daily maxima gradually converging towards noon to reach unimodality with a maximum at 11 AM in October. Thereafter, bimodality reappears and continues, becoming only feebly evident as an apparent residual in the essentially unimodal inverted cycle of January which like the succeeding month, February, has a 7 PM maximum.

The study of the carrot revealed striking similarity of its major mean cycles with those of the potato. Figure 4 shows the mean % noon deviation from linear trend for each of the eight months. Like the results obtained with the potato for the same calendar period, this passed from an early-fall higher value, through a winter minimum and back to a higher spring value. Fewer data were available during the first three months, hence the errors were larger. The range was less than for the potatoes. Figure 5, A and B solid curves, compares the mean 8-month daily cycles for the carrot and potato, and the average cycles for the two fall and two spring months (dashed curves) as compared with those for the four intervening colder months (dotted curves), The similarities of these two widely different kinds of plants and plant portions (roots vs. stems) for the same periods, in the % amplitudes of the fluctuations, in the times and the changing times with time of year of the primary maxima, and in the times of secondary, or incipient, maxima, are strikingly apparent from the figures.

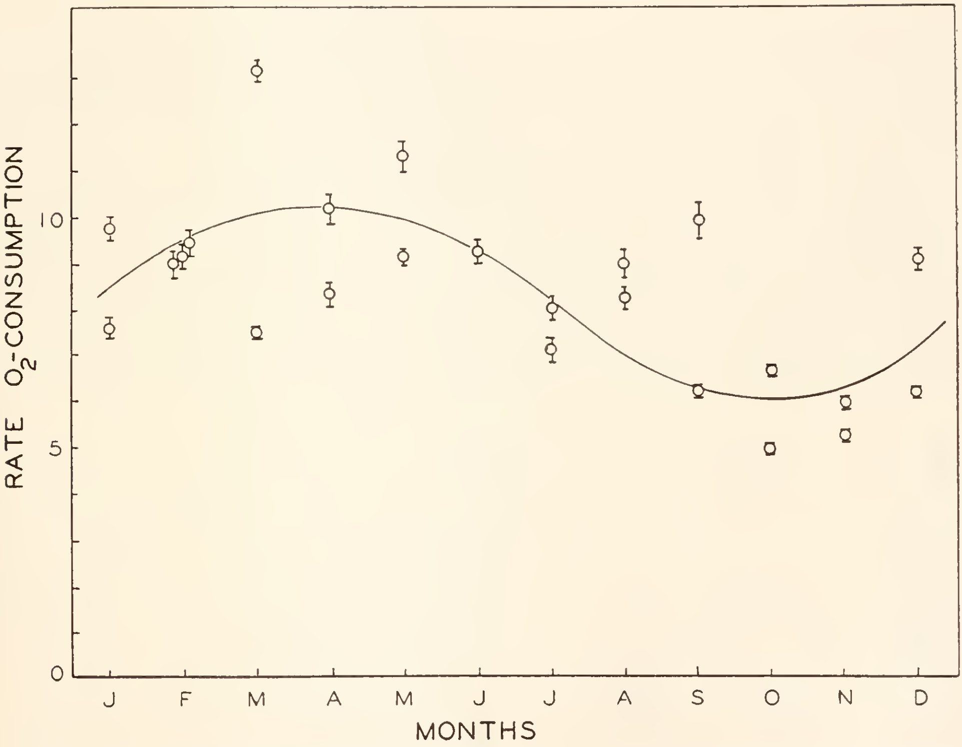

FIGURE 6. The relationship between mean rates of O_2-consumption and each of the 24 months studied during 1956, 1957, and 1958, and time of year. Standard errors of the means are indicated.

A second kind of annual cycle appears also present in the data. This is in the mean daily metabolic rates. In Figure 6, are to be found the mean monthly rates of O_2-consumption, in arbitrary units, for each of the 24 months of study, together with their standard errors. Two conclusions are evident from the figure: (1) The maximum rate of O_2-consumption occurs in the April–May period of the year and minimum rate in October–November. The rate for the former period ap-

Table I

Signs of the average monthly correlations of the 6 AM deviations from linear trend with the mean 2–6 AM rate of barometric pressure change

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Year 1		−	−	−	+		+	+	+	+	−	−
Year 2	−	−	+	+	+	+	+	+	+	+	−	−
Year 3	−											

proaches twice that of the latter. (2) The mean rates for corresponding months of the two consecutive years may be quite significantly different from one another, suggestive of a specific, *time-environmental* factor involved in an exogenous regulation. There is nothing in these data to suggest other than that the mean form of this annual cycle will ultimately be found essentially sinusoidal.

In view of the correlations highly significantly different from zero earlier reported (Brown, 1957a) to exist between the 5–6–7 AM mean deviations (without sign) in O_2-consumption from the daily means and the mean 2–6 AM rates of barometric pressure change, this relationship was examined for the two-year period involved here. Three-hour values of O_2-consumption centered on 6 AM were recorded as deviations from linear daily trend, and three-day moving means calculated. These were correlated with comparable three-day moving means of the av-

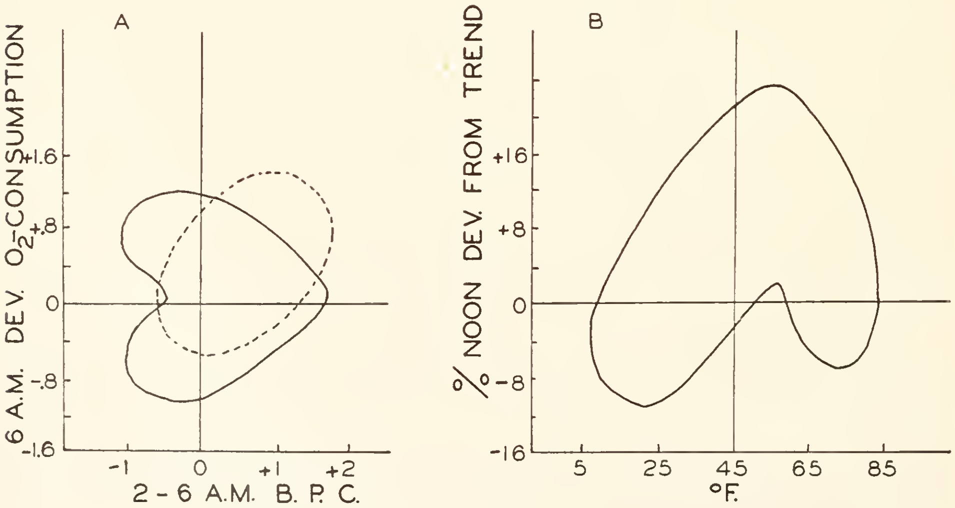

Figure 7. A. Solid line: An outline of the general form of the scatterplot between mean 2–6 AM rate of barometric pressure change and the 5–6–7 AM mean deviation of rate O_2-consumption from daily trend for the same day during the "colder" months (see text). Broken line: The same for the "warmer" months. Data for both involve three-day moving means. The two patterns together include 98% of all points. B. An outline of the form of the scatterplot (97% of all points) between % noon deviations from linear trend in potatoes in constant conditions and concurrent outdoor air temperature, taken from data of 149 non-overlapping three-day averages.

erage 2–6 AM rate of barometric pressure change, for the corresponding days. It should be emphasized that only a single value was used for each day for each phenomenon; hence, this did not involve a correlation of parallel daily cyclic trends. A positive coefficient, highly significantly different from zero, was obtained. This correlation, as one would anticipate in view of the essentially aperiodic, large climatic barometric pressure changes, rapidly drops to insignificance as one correlates

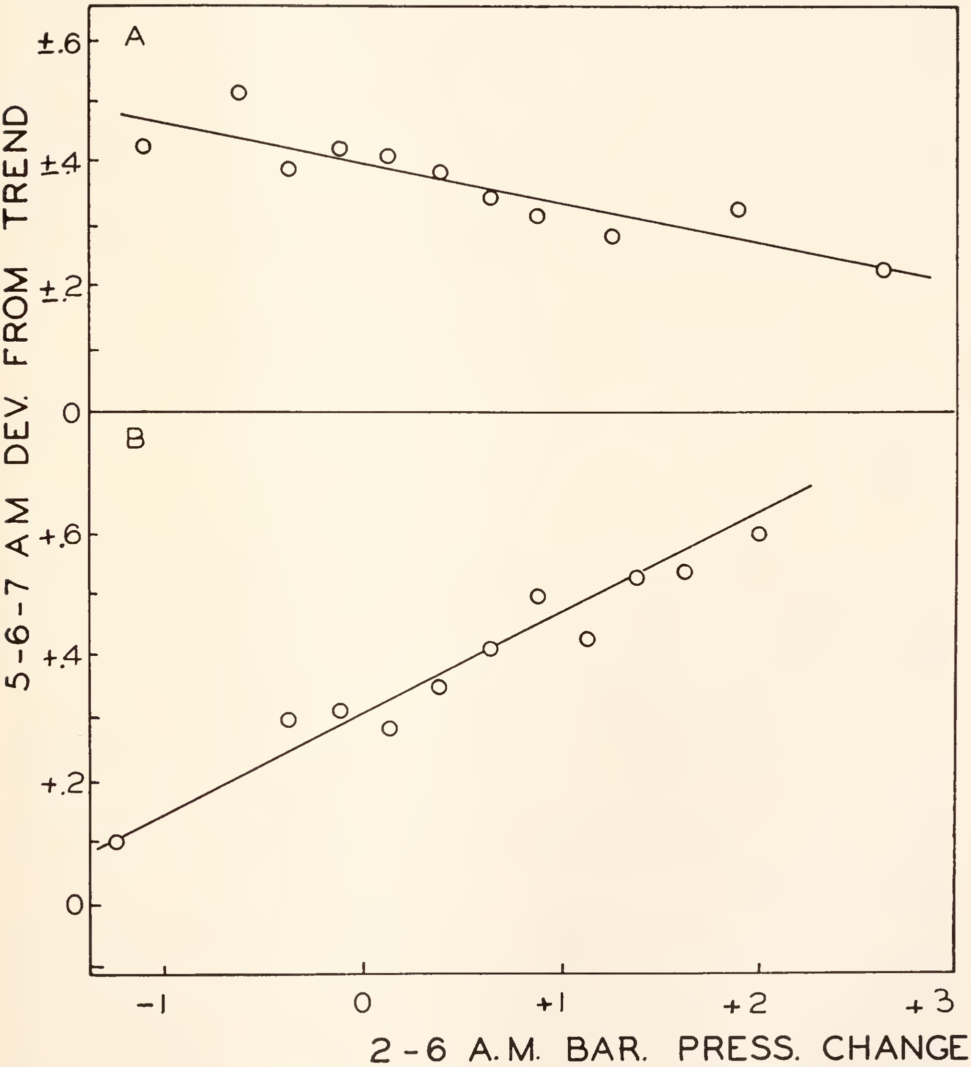

FIGURE 8. The regressional relationship of the average three-hour rate of O_2-consumption of the potato centered on 6 AM, and expressed as deviation from linear daily trend, on the average rate of barometric pressure change during the 2–6 AM interval for the same morning for the colder months of the year (see Table I). $P < 0.005$. B. The relationship comparable to that in A, but for the warmer months of the year (see Table I). $P < 0.001$.

in increasing lag on lead relationships up to two to three days (Brown, 1957a) indicating a direct response of the organisms to some pressure-change-correlated external variable. But this relationship was found to contain a characteristic sign-change twice a year as seen in Table I. In this table, a dash indicates those months in which there was a negative correlation between the rate of the 2–6 AM barometric pressure change and the 6 AM deviation, *without sign*, from linear trend. The form of the scatterplot relationship for the 299 days of this negative period is outlined by the solid curve (encloses 91% of the points) in Figure 7, A. The regressional relationship of the deviation in O_2-consumption, without sign, upon pressure change is seen in Figure 8, A. During the + months, on the other hand, there was a positive correlation between the rate of 2–6 AM barometric pressure change and the 6 AM deviation in O_2-consumption from linear trend. Ninety-one per cent of the 389 days in a scatterplot of the relationship for these months fell within the broken curve of Figure 7, A (98% of all 688 daily points + or − months fell in the areas prescribed by both the solid or broken lines). The regressional relationship of O_2-consumption on pressure for the + months, with sign, is seen in Figure 8, B.

TABLE II

Signs of the average monthly 2–6 PM change in barometric pressure

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Year 1		+	+	+	−	−	−	−	−	−	+	+
Year 2	+	−	+	−	−	−	−	−	−	−	+	+
Year 3	+											

Since in the warmer, positive, months of the year, the overwhelming mass of the deviations was +, it was not possible to find any real difference between the correlations whether the deviations were treated with, or without, sign. In the colder, negative, months, on the other hand, about half of the deviations were negative, and the range of the latter as great as for positive deviations.

In view of the earlier report (Brown, 1957a) of comparable correlations between the 6 PM deviation in O_2-consumption from daily mean values in potatoes, and the afternoon rate of barometric pressure change, and also correlations with the mean daily pressure of the second day thereafter (Brown, Webb and Macey, 1957), the former relationship including signs, it is of interest to compare the annual cycle in the sign of the average 2–6 PM barometric pressure change. These are seen in Table II.

The similarities between Tables I and II suggest that this aspect of organismic annual cyclicity, involving the mean forms of daily cycles, might in some manner be caused by a factor whose daily fluctuation reflects the annual cycle in form of the well-known mean daily tidal atmospheric pressure cycles. In these daily pressure cycles, the time of the morning maximum remains relatively fixed throughout the year at 9–10 AM, but the afternoon, major minimum of the day gradually shifts from about 2 PM in winter to about 7 PM in summer. This last is the basis for the sign changes in Table II. Thus, any pressure-correlated effective external physical factor could provide such an annual cycle in the daily cycles as that described herein.

Another clearly evident correlation is seen in the relationship of the % noon deviation from linear trend in the potato to the concurrent outside mean daily air temperatures.[3] A two-year study of the comparison of non-overlapping three-day periods of air-temperature and of noon deviation of the daily cycles in constant conditions yields a scatter plot relationship as illustrated in Figure 7, B. The line includes 97% of the 149 values. The regressional relationship of noon-deviation of the potatoes on temperature (using 5° F. class intervals) is illustrated in Figure 9. The relationship seems adequately described as a linear one, but with a sign change near 57.5° F. Calculation of the coefficient of correlation for noon deviation in O_2-consumption with the ± deviation in temperature from 57.5° F., yielded a value of -0.51 ± 0.049. This clearly indicates that the external factor responsible for the 24.0-hour cycles of metabolism is correlated in its fluctuations with air temperature, resulting in a condition where a spurious organismic Q_{10} of cycle amplitude of more than 3 could be apparent (*e.g.*, in range 32.5° to 52.5° F.). This provides another piece of information which will probably lead eventually to identification of the still unknown external factor responsible for the organismic basic periodisms.

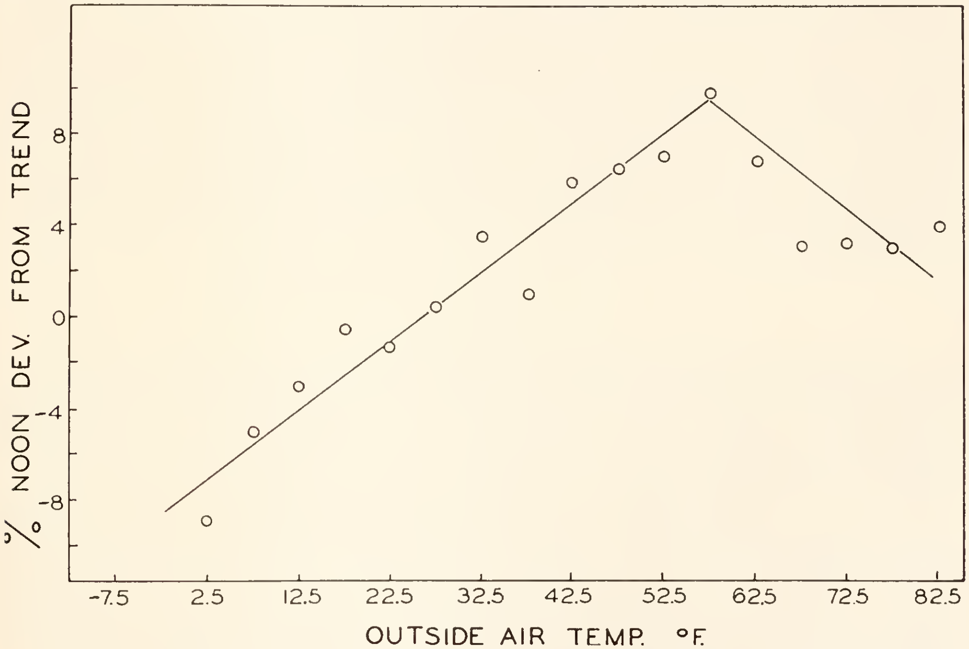

FIGURE 9. The regressional relationship of the noon deviation in O_2-consumption in the potato, expressed as % deviation from daily linear trend on simultaneous outdoor air temperature.

That the relationship to temperature is rather substantial, is given further support in that the regressional relationship of noon deviation on temperature exhibited a sign change about 57° F. in the first year, 1956–57, just as it did again in the second, 1957–58, despite the fact that in 1956 there was no clear *absolute* summer decline in amplitude of the mean monthly cycles (Fig. 2) as was found in 1957. Also, during the winter months the correlations are observed (Fig. 9) to continue in the same linear relationship even at temperatures lower than the lowest mean monthly ones in the two years involved in this correlation (29.4°, 28.7°, 18.8°, 27.3°, and 26.3° F.) which averaged about 26° F. These last facts suggested intra-month significant temperature correlations which were borne out by investigation of the correlations using the data of the five coldest months now expressed as deviations from monthly means. The correlations continued in temperature ranges well exceeding any mean month-to-month difference.

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[3] These data were generously provided to me by the Chicago Office of the U. S. Weather Bureau.

Again, using the data for both of the two years, the transitional months, April, May, October, with a mean temperature of 54.8° F. (47.2, 60.5, 60.8, 49.5, 58.8, 52.0), expressed as deviations from monthly means, the critical temperature for sign change was again quite apparent. Finally, employing the warmest months of the year, June, July, August, and September with a mean temperature of 71.1° F., (72.9, 74.1, 65.0, 71.2, 76.4, 73.4, 64.2), there was a suggestion of the existence of a second sign change with again a positive correlation at the higher temperature. The number of high-temperature days was insufficient, however, to enable resolution of this last point.

Discussion

From the foregoing results it is evident that potatoes, and apparently the carrots too, display a quite reproducible mean solar daily cycle provided adequately long periods of time are used to render random the influences of such modulating longer cycles as a lunar day (Brown, Freeland and Ralph, 1955), a synodic month (Brown, Freeland and Ralph, 1955; Brown, Bennett and Webb, 1958), and, in this report, a low amplitude apparent sidereal day, and an annual cycle. These described mean solar-day cycles are obviously of quite precise 24.0-hour frequency, and adequate evidence is at hand to be assured, beyond all reasonable doubt, that these have their frequency exogenously determined. This last conclusion is assured in part through the well-known knowledge that there are solar-day tidal rhythms of atmospheric pressure, together with the fact that the living organism has access to information of them through its responses to the day-to-day, essentially random, weather-induced, disturbances in their regularity. That the factor influencing the organism is not pressure itself, is evident from the fact that these and other experiments have involved organisms maintained for long periods in constant pressure. The external factor which is involved appears to have its primary action upon the organism at the times correlated with the early-morning rise in barometric pressure and the afternoon fall. These would presumably be the times of most rapid change in physical factors fluctuating with the day-night cycle, and hence be the times of their maximal stimulative effectiveness.

As pointed out earlier in this report, the presence of the well-known annual change in the form of the daily, tidal, barometric pressure cycles, and the described response of the organism in the late afternoon to a pressure-correlated external variable would have led to the prediction of the occurrence of an annual cycle in the form of the daily cycles. Such a prediction has been fulfilled in this study. This adds still further, therefore, to the assurance that the forms of the daily basic metabolic oscillations in living organisms are exogenous.

Since background radiation, too, possesses good mean solar-day cyclicity, and the organism follows the essentially random fluctuations in its cycle amplitudes from day-to-day (Brown, Shriner and Webb, 1957) very safely beyond what would be expected through chance, when and only when contemporary data are correlated, this constitutes a third line of evidence for exogenous origin of mean daily metabolic cycles.

The existence of an annual cycle in the potato in constant illumination, temperature, pressure, etc., was reported earlier for fluctuations in linear daily trend (Brown, 1957c), as were also synodic monthly cycles of this parameter (Brown, Bennett and Webb, 1958). In this paper there is described an apparent annual cycle in basic metabolic rate, a cycle which appears to be of simple sinusoidal character with maximum in April–May and minimum in October–November. This cycle involves an approximate doubling of rate in passing from minimum to maximum values in the annual cycle. Comparable, synodic monthly, cycles in metabolic rate in potatoes (Brown, Bennett and Webb, 1958) involved, as the average during a year of study, about a 15% increase from minimum (new moon) to maximum (third quarter) values. By further comparison, the amplitude of the daily cycles, though undoubtedly artificially depressed through the use of the seven-hour weighted moving means, displayed about a 3.7% increase from midnight minimum to 6 PM maximum values.

The regressional relationship of amplitude of the daily cycles on mean daily temperature for three-day periods (Fig. 9), with its coefficient of determination of about 0.26, and its critical temperature for sign-reversal, together with the earlier barometric-pressure-change reversing correlation, suggests again the exogenous origin of this daily cycle period, and, at least in large measure, also cycle form. This is especially true, since the relationship to temperature seems to persist into the weather-correlated, intra-month, temperature fluctuations.

In examining Figure 9 and noting the relationship of cycle amplitude to temperature, and recalling that the mean daily temperature range is about 16° F., with not very uncommonly single days with ranges up to 25° to 30° F., one is tempted to postulate that the factor that is responsible for transmitting to the organism in "constant conditions" information on outside air-temperature, is, through its temperature-correlated fluctuations alone, contributing importantly to the 24-hour periodic metabolic fluctuations themselves. In support of this hypothesis is the rough similarity in the average forms of the annual fluctuations in the amplitudes of ground-level daily temperature change and metabolic cycles. Both, as average for the two years, showed lowest values in the coldest winter months and highest values in late spring and late summer to early fall, with a summer amplitude reduction. The relationship between these two phenomena is seen in Figure 10. The October peak, so conspicuous for the metabolic cycles, is much less evident for the temperature changes.

For each year this relationship between these two phenomena appeared to trace out general ovoid form. The two-year mean month-by-month relationship is shown by the numerals 1 (January) through 12 (December), and the dotted ovoid curve roughly traces their course. It is interesting to speculate that this difference between the organismic and temperature annual cycles may find its explanation in the changing natural smog content of the atmosphere. The terpenes, volatilized from plants, polymerized by the ultra violet light from sun, reach a maximum in October (personal communication from Professor F. W. Went). This smog may, through influencing the amount of heat absorption from sunlight, produce in October the highest amplitude daily temperature changes of the year at levels in the atmosphere where temperature changes produce greatest influence upon the factor directly affecting the organism. One process, known to be temperature-dependent, is the rate of spontaneous decay of cosmic-ray-derived mesons. The larger the atmospheric depth involved in the temperature change, in this instance, the larger would be expected the temperature influence.

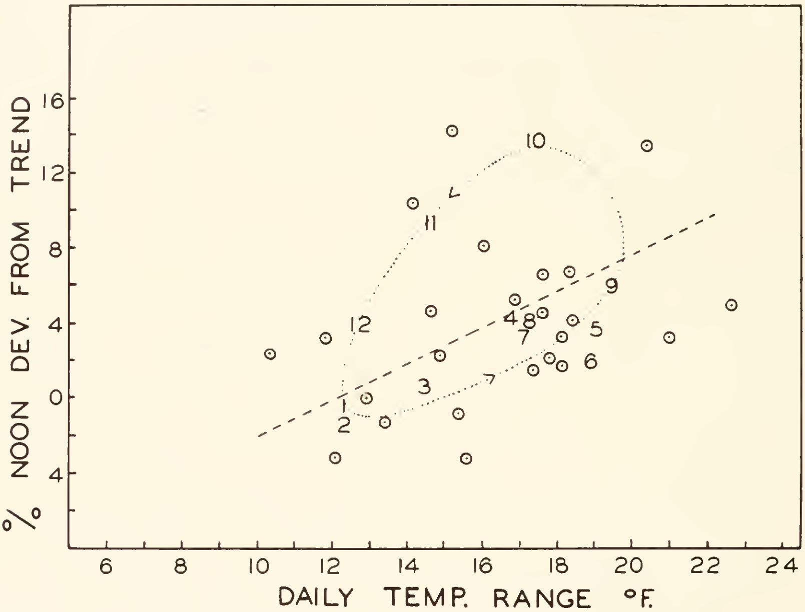

FIGURE 10. The relationship between mean monthly daily temperature ranges and the mean monthly noon deviations in O_2-consumption from daily trend. The Arabic numerals indicate the mean monthly relationships for both of the years, with January as 1, February as 2, March as 3, etc.

Now that it appears true beyond reasonable doubt that the living organism, as part of its cyclic geophysical environment, is exhibiting metabolic cycles with the natural solar day, lunar, and annual periods, the question arises as to what the relationship of these metabolic cycles might be to the well-known endogenous physiological and behavior cycles which have been abundantly described, especially during the past fifty years for species representing the gamut of the animal and plant kingdoms. Those studied experimentally have included especially the sleep-movements of plants, the spontaneous motor activity of numerous animals, physical and chemical adaptation of compound eyes, integumentary color changes, eclosion in flies, learned periodic behavior in insects and lunar-tidal rhythms of color-change and of motor activity. These cycles are clearly endogenous in most instances since the forms and phase relationships of the cycles relative to external physical ones may be modified by appropriate treatment of the organism and will persist for a few, and often many, cycles. The endogenous cycles also may gradually drift away from their initial phase relationships when placed in constant conditions away from cyclic light and temperature changes. The rate of drift is characteristically a function of the constant illumination, or constant-temperature, level; brighter light behaves usually like higher temperature. But in some other instances the cycles show no measurable drift over long periods, behaving in their precision, therefore, more like the mean metabolic cycles. Also, cycles of quite other periods than the natural daily ones may be directly induced by light, for example, but the organism thereafter placed in constant conditions reverts at once to daily cycles.

The characteristic of essential temperature-independence of the cycle-frequencies, whether one deals with solar-day, lunar, or annual ones, made it rather improbable that the basic frequency-determining mechanism was endogenous. However, one hypothesis that has been rather widely entertained bears the assumption of the possession by the organisms of accurate, fully autonomous, endogenous biological clocks timing all the natural period lengths. Although the majority of investigators have always cautiously dissociated the possible clock mechanism from the endogenous physiological cycles studied and whose frequencies they appeared to regulate, some of the more recent supporters of this hypothesis of an autonomous endogenous clock have uncritically identified the fundamental clock system with each of the various observed endogenous and labile physiological cycles studied. On the basis of this quite unjustified assumption, the postulated clock is then usually considered to have all the properties demonstrable for these clock-regulated, and undoubtedly endogenous, cycles.

None of the several hypotheses suggested for the independent biological clock, however, have satisfactorily resolved the problem of the determination, in an essentially temperature-independent manner, the exact intervals of the turning points in the persisting cycles. So-called endogenous rhythms with single cycles ranging from 24 hours to a year in length cannot readily be conceived in terms of any fully autonomous mechanism based upon the reaction kinetics of any biochemical, or biophysico-chemical, systems with which we are now familiar.

An alternative hypothesis, advanced by Brown (1957b), proposed that the basic mechanism of temperature-independence of the frequency of biological rhythms in "constant conditions" involves the operation of a cyclic exogenous stimulus operating upon a responsive protoplasmic system, and giving rise in all cells to systematic fluctuations containing all the major natural periodisms of the external environment. These could then be readily used by the organism in timing its endogenous cycles. The latter could be considered as bearing any pre-set, fixed lag or lead relationship, or even possess a smoothly and continuously drifting relationship, thus providing endogenous cycles of frequencies differing slightly in lengths from 24 hours. The often-reported small influence of light and temperature level on cycle length in constant conditions can well operate through influencing the coupling mechanism between the exogenous basic clocks and the endogenous organismic rhythms.

We still do not know whether the exogenous daily clocks operate on a universal or local-time basis. It is even quite possible that both kinds of cyclic elements are present, each through correlation with other geophysical factors possessing one or other of these two cyclic characters, *e.g.* atmospheric electrical potential change (universal time); tides of atmospheric pressure (local time). The existence, however, of exogenous daily cycles has been demonstrated by means of local-time-related, aperiodic phenomena, which are in good measure superimposed on the local-time-related tides of the atmosphere.

It is evident that the solar daily cycle described in this research is clearly of solar-day rather than sidereal-day length. The possible existence of a cycle of the latter period-length was investigated. The existence of one was suggested by the annual cycle involving a positive to negative deviation about 1 AM-to-midnight linear daily trend (Fig. 2), and by the apparent gradual shifting of the solar-day maximum across the day (Fig. 3). This day, being 3.93 minutes shorter than the solar day, would be expected to scan the solar day almost exactly once each year. Similarities in the form of mean-metabolic cycles with cosmic radiation ones (Brown, Webb and Bennett, 1958) and of metabolic cycle amplitude with background radiation cycle amplitude (about half of this radiation is thought to be of cosmic-ray-origin), both give further likelihood for the existence of such a period-length in the living organism. The mean, apparent sidereal-day cycle which was found showed an amplitude of less than 2%. However, in view of the extreme closeness of the period of the year to the length of the cycle of periodic reinforcement of solar-day with sidereal-day cycles, many years of data would obviously be required to resolve the problem of the relative roles of the sidereal day and annual cyclicities in the production of the cycle depicted in Figure 1, B.

It should be emphasized that the low correlations obtained in this study in no manner imply that the correlations with the effective agent would be similarly small. Correlations between consecutive values in parallel cycles of the same frequencies would be expected to be, and are, much higher. The latter, however, could not be used to demonstrate the dependence of one cycle upon another or even on a factor correlated with the second. The cycles of the effective factor may be substantially more regular, and its correlations with organismic metabolism of a much higher order.

SUMMARY

1. Oxygen-consumption was monitored almost continuously in potatoes, *Solanum tuberosum,* in constant conditions, including pressure, for more than two years. A paralleling 8-month study of O_2-consumption in carrots, *Daucus carota,* was also made.

2. The potatoes showed an essentially bimodal mean daily cycle with an average amplitude of the major maximum of 3.7%. The cycles for the two years taken separately were very closely similar.

3. The daily cycle exhibited an annual cycle of form change, with cycles unimodal, inverted, with a 7 PM maximum in February and unimodal with an 11 AM maximum in October. The intervening months yielded bimodal cycles, with graded transitional forms.

4. The daily cycle and its annual fluctuation in the carrot resembled in great detail those obtained concurrently for the potato.

5. An annual cycle in average daily rate of O_2-consumption was found in the potato. The cycle was essentially sinusoidal with minimum in October–November and maximum, the rate about doubled, in April–May.

6. Throughout the two years the 5–6–7 AM deviation in O_2-consumption from linear daily trend was always correlated with the 2–6 AM mean rate of barometric pressure change for the same morning. The sign of this correlation exhibited a characteristic change twice each year, once in the spring and again in the fall.

7. For the two-year period of study the amplitude of the daily cycles showed a linear correlation with the concurrent outside air-temperature, with the sign of the correlation reversing about 57.5° F. With temperature expressed as deviation from 57.5° F., the coefficient of correlation was -0.51 ± 0.049.

8. The data suggest the existence of a rhythmic component of sidereal-day length in the potatoes. Problems in its final resolution are discussed.

9. The evidence points quite conclusively to the possession by the organism, even in so-called "constant conditions," of environmentally imposed oscillations of the natural, daily and annual periods.

10. The fluctuations in the still unidentified, external effective factor appear importantly influenced by, and may possibly even in some measure determine, meteorologic changes of temperature and pressure.

11. The significance of these findings for the problem of the mechanism of the basic daily and annual biological "clock" regulating in "constant conditions" the well-known endogenous organismic rhythms is discussed at some length.

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SULFURIC ACID IN DESMARESTIA

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Several species of *Desmarestia* accumulate acid in their tissues (Blinks, 1951). Kylin (1938) first considered malic acid to be responsible for the low pH values of expressed *Desmarestia* sap. But Wirth and Rigg (1937) and Meeuse (1956) identified the acid as sulfuric. The titration curve of *Desmarestia* sap is that of a strong acid; the pH may be as low as 0.78 and sulfate ions are present in high concentration.

In a review, Blinks (1951) pointed out a correlation between the thickness and relative surface area of three California species of *Desmarestia* and their acid content. The pH of the sap of *Desmarestia munda,* a species with thick blades, is one or less; *D. herbacea* has somewhat narrower and thinner blades and its sap has a pH of two to three; and *D. latifrons,* the most delicately branched of the three species, is only weakly acid with sap of approximately pH five. Blinks also stated that the locality of the acid within the algal tissues is unknown, and suggested that it may occur in the cell vacuoles. He found that the outer cell membranes were injured by acid, and thus it seems unlikely that the acid is free in the cytoplasm.

Kylin (1938) stained *Desmarestia* cells with the vital dye, brilliant cresyl blue, and observed that the vacuoles appeared purple. He concluded that the vacuolar contents were alkaline, because the dye changes from blue to reddish-violet from pH 7.0 to 7.5. He apparently overlooked that fact that the dye also changes from blue to purple from pH 1.0 to pH 0.7. So his conclusion that the vacuoles are alkaline may be questioned. It seems more likely that the vacuoles are strongly acidic. This report provides additional evidence that hydrogen ion accounts for a large part of the vacuolar cation content of the acid-accumulating species of *Desmarestia.*

METHODS AND MATERIALS

Desmarestia munda and *D. herbacea* were collected at Pebble Beach, California. For comparison, two non acid-accumulating brown algae, *Egregia laevigata* and *Dictyoneurum californicum,* were also collected. The plants were kept in running sea water in the laboratory before use.

The blades were washed free of sea water in 0.6 *M* sucrose for five minutes and extracted with five per cent trichloracetic acid. Sodium and potassium contents of the extracts were determined by flame photometry. Total acidity was determined by titration of hot water-extracts of the algae.

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Amounts of adsorbed cations were assumed to be the same for living and dead tissues, hence the values determined for killed tissues were subtracted from those for living tissues. The difference may represent the intracellular cation content. The apparent osmotic volume of the tissues (Briggs and Robertson, 1957) was estimated as the difference in sucrose apparent free space of living and killed tissues (Eppley and Blinks, 1957). The tissues were killed by boiling or by soaking in fifty per cent ethanol. Ion concentrations are expressed on the basis of the estimated osmotic volumes.

Tissues were placed in a Waring Blendor in 0.8 *M* "tris" buffer (tris hydroxymethyl amino methane-HCl), initial pH approximately 8.5, blended for five minutes, and filtered through cheesecloth for use in methylene blue reduction experiments.

TABLE I

The effects of inhibitors of selective permeability on the rate of acid-loss into the medium of Desmarestia munda tissues as determined by the time required for color change of methyl orange. Tissues in aerated sea water

Expt. 1	Minutes for color change	Expt. 2	Minutes for color change
no inhibitor	>60	no inhibitor	>60
p-chloromercuribenzoate	12	p-chloromercuribenzoate	14
(0.0005 *M*)	16	(0.0005 *M*)	17
NaCN (0.001 *M*)	10		
	12	iodoacetate (0.002 *M*)	2
			1

RESULTS

Several experiments indicate that the acid is within the cells, yet not free in the cytoplasm. Tissue extracts were capable of reducing methylene blue with a variety of substrates, under nitrogen, only if buffered near neutrality. No activity was noted in preparations in which the pH of the extract was less than five or if the tissues were homogenized in unbuffered sea water. Rates of dye reduction were somewhat greater in the presence of ribose and aspartate than with other substrates. Some activity was also present in buffered extracts with no substrate added, but quantitative studies were not made.

In other experiments, discs of the thalli, cut out with a two-cm. cork borer, were tested for acid loss in sea water in the presence and absence of inhibitors. Samples of five or ten discs were placed in ten ml. of sea water containing a drop of methyl orange, and the time required for a color change of the indicator was recorded. The rate of acid loss was much greater in neutralized sea water in the presence of 0.0005 *M* p-chloromercuribenzoate, 0.001 *M* sodium cyanide, and 0.002 *M* iodoacetate than in sea water alone (Table I).

Rates of acid loss in dinitrophenol (0.005 *M*) were determined by measuring the pH of the solution. The curve resulting from a plot of pH against time (Fig. 1) suggests an autocatalytic reaction. This autocatalytic injury is implicit in Blinks' (1951) description of the rates of carotenoid color change in *Desmarestia* as the alga dies.

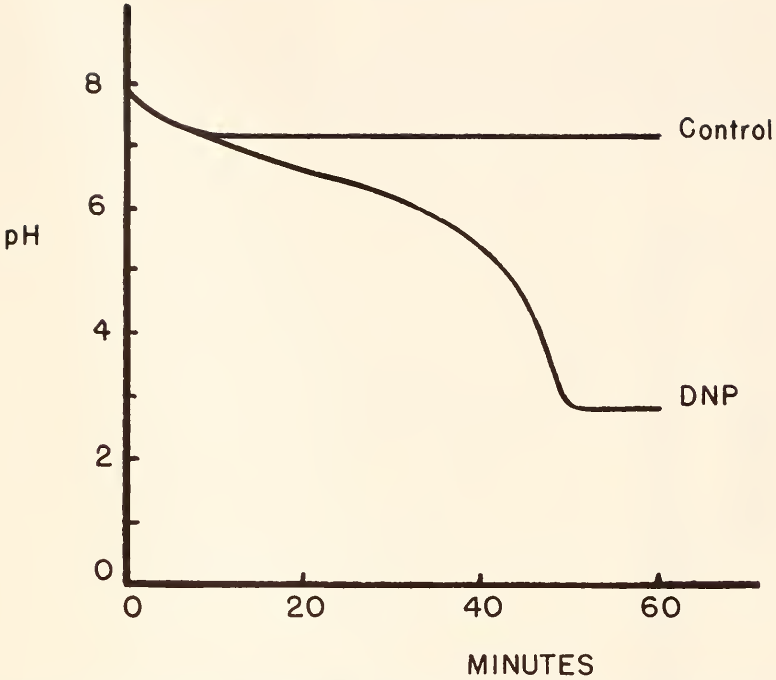

FIGURE 1. Acid release by *Desmarestia munda* tissues in sea water (control) and in sea water containing 0.0005 *M* dinitrophenol (DNP).

On accumulation of the dye, brilliant cresyl blue, the vacuoles of *D. munda* and *D. herbacea* are stained purple in confirmation of Kylin's results (1938). However, we feel the color to be indicative of the change at pH 1.0–0.7, rather than 7.0–7.5.

If the acid is localized within the vacuoles, one might expect the cations normally found in the vacuoles of brown algae to be replaced by hydrogen ions. In *Egregia laevigata* and *Dictyoneurum californicum* (Table II) potassium is the most abundant cellular cation measured. It occurs at a concentration approximately isotonic with sea water. In *D. munda* about 75 per cent of the potassium is replaced by hy-

TABLE II

Potassium, sodium, and hydrogen ion contents of Desmarestia munda, D. herbacea, and two non-acid-accumulating species of brown algae. Values are corrected for the ion contents of killed tissues and represent averages of four determinations. Units milli-equivalents/liter estimated cell osmotic volume

Alga	est. cell. osm. vol.	H	K	Na	Sum
Desmarestia munda	84%	438	148	—	586
Desmarestia herbacea	69%	254	234	13	501
Dictyoneurum californicum	63%	—	523	21	544
Egregia laevigata	71%	—	542	45	587

drogen, and about 50 per cent is replaced in *D. herbacea* (Fig. 2). The reciprocity of potassium and hydrogen ion concentrations agrees with the above mentioned expectation. The approximation of the total cation concentration among the four brown algal species to that of sea water suggests that most of the cation content is accounted for, although magnesium and calcium were not measured and may be present.

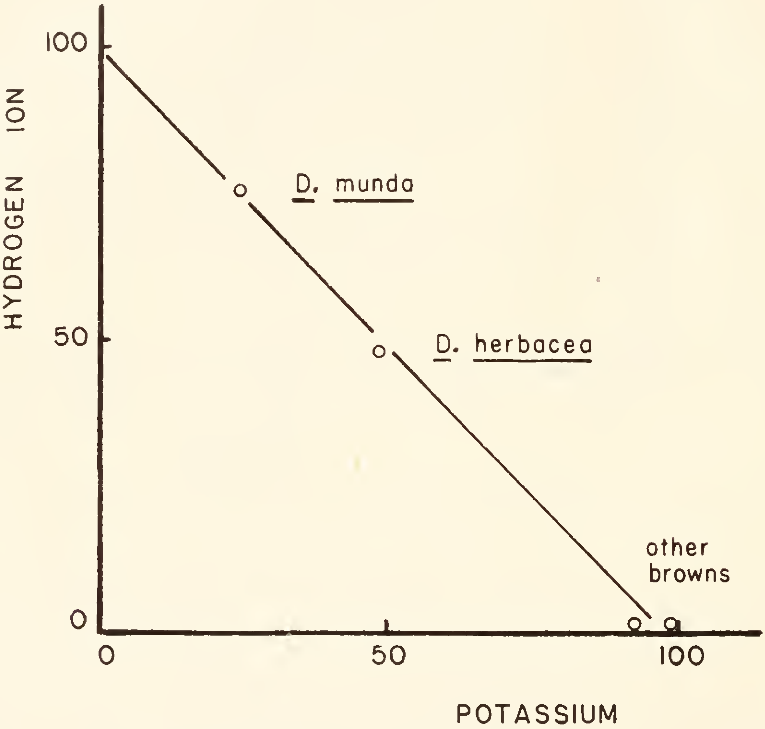

FIGURE 2. Hydrogen and potassium ion contents of *Desmarestia munda, D. herbacea,* and two other brown algae: *Egregia laevigata* and *Dictyoneurum californicum.* Units: per cent of total cation content determined.

The binding of large amounts of sodium by dead tissues was detected. This may represent adsorption of the cation to the carboxyl groups of alginic acid, a structural polysaccharide of the brown algae (Wasserman, 1949).

DISCUSSION

The vacuoles of *Desmarestia* contain sulfuric acid in amounts up to 0.44 N, in *D. munda.* Direct evidence for this view is the purple color of brilliant cresyl blue accumulated by the vacuoles of *D. munda* and *D. herbacea.* Indirect supporting evidence includes the following: 1) The acid is lost more rapidly on exposure of tissues to inhibitors which abolish selective membrane permeability than it is in the absence of such inhibitors. In this group are sodium cyanide, iodoacetate, p-chloromercuribenzoate, and dinitrophenol. 2) The autocatalytic release of acid in the presence of dinitrophenol suggests that extra-vacuolar acid injures the cells, causing an increasing rate of acid release. 3) Oxidative metabolism is sensitive to high hydrogen ion concentrations as evidenced by the inability of tissue extracts to reduce methylene blue in unbuffered suspensions. 4) The reciprocity of potassium and hydrogen ion concentrations among the brown algae tested suggests that hydrogen replaces potassium as the most abundant cellular cation in *D. munda,* and that about one-half of the potassium is replaced in *D. herbacea.*

The tonoplasts of *Desmarestia* cells must be quite unique in their resistance to acid injury, and in their permeability characteristics. A hydrogen ion concentration gradient of about 10^7 is apparently maintained between the vacuolar sap and sea water. However, the sea water is probably not the "substrate" for hydrogen ion accumulation. Metabolically produced hydrogen in the cytoplasm may well be the source for vacuolar accumulation. Efforts to leach the acid from the cells so that the progress of acid reaccumulation could be studied have not been successful. The cells are killed as the acid is released.

The production of hydrogen ion due to anaerobic conditions in the interior cells of massive species of *Desmarestia* may explain Blinks' (1951) observation of a correlation between tissue massiveness and acid content. The interior cells of *D. munda* are much larger, contain fewer plastids, and show a greater percentage of purple vacuoles, on staining with brilliant cresyl blue, than the peripheral cells or the cells of *D. herbacea.*

The high acidity of *Desmarestia* cells may limit the vertical distribution of the alga in the intertidal zone. Because injury spreads so rapidly when water circulation is poor, it seems reasonable that the acid-accumulating species must be confined to regions of constant water circulation. *Desmarestia herbacea* occurs below the lowest-lower-low-water tide mark (Doty, 1946) and *D. munda* is limited to the lower portion of the intertidal zone (Smith, 1944).

SUMMARY

1. Brilliant cresyl blue accumulates in the vacuoles of *Desmarestia munda* and *D. herbacea* and the accumulated dye appears purple, indicating that the pH of the vacuolar sap is less than 1.0 or greater than 7.5. However, the expressed saps of these two brown algae have pH 1.0 or less and about 2.0, respectively. The outer cell membranes are injured by the low pH of the sap and methylene blue is not reduced by tissue homogenates at such low pH values.

2. Sodium cyanide, dinitrophenol, iodoacetate, and p-chloromercuribenzoate induce the release of acid from the cells, in which potassium, normally the cation most abundant in brown algal cells, is largely replaced by hydrogen. In *D. munda* hydrogen accounts for 75 per cent of the intracellular cation content. Tissue sodium is largely bound and contributes little to the cellular cation content.

3. The simplest interpretation of these data is that the acid is localized within the vacuoles of *Desmarestia* cells.

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THE SENSITIVITY OF ECHOLOCATION IN THE FRUIT BAT, ROUSETTUS

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Moehres and Kulzer (1956b) have reported that among the Megachiroptera (Old World fruit bats and flying foxes) the genus *Pteropus* orient visually while *Rousettus aegypticus* orient visually but also echolocate. Six additional megachiropteran genera, *Eidolon, Cynopterus, Ptenochirus, Lissonycteris, Eonycteris,* and *Macroglossus,* have all proved, like *Pteropus,* to orient visually and not acoustically. Observations of two additional species of *Rousettus, R. amplexicaudatus* and *R. seminudus* as well as *R. aegypticus,* have confirmed Moehres and Kulzer's conclusions (Novick, 1958). *Rousettus* generate clicks by movements of the tongue and emit these through the open corners of the mouth (Kulzer, 1956) rather than producing sounds laryngeally as do the Microchiroptera (Griffin, 1946, 1952; Novick, 1955; Griffin, 1958).

As far as is known at present all of the Megachiroptera except *Rousettus* are helpless in total darkness. *Rousettus* apparently make use of vision and/or echolocation depending upon the light conditions, the difficulty of their flight path, and the type of flight required (take-offs and landings, for example). The echolocation system used by *Rousettus* has almost surely evolved independently of the system employed by the Microchiroptera. Furthermore, it resembles in design the system serving much the same purpose in the cave-dwelling birds, *Steatornis* and *Collocalia.* The isolation of these three natural sonars in single genera, their simple designs, and their facultative employment (all three genera orient visually in adequate light) make it seem likely that they are recent developments compared with undoubtedly ancient microchiropteran echolocation systems. There is, therefore, considerable interest in comparing the effectiveness of the echolocation system of *Rousettus* in the detection of small objects with that achieved by the Microchiroptera, especially some carefully studied species of the families Vespertilionidae and Phyllostomatidae (Curtis, 1952; Griffin and Novick, 1955; Grinnell and Griffin, 1958).

Since the orientation clicks of *Rousettus, Steatornis,* and *Collocalia* are clearly audible to man, they obviously contain more energy at frequencies below 20 kc than do the orientation pulses of most of the Microchiroptera. The principal component in *Rousettus* clicks is between 12 and 18 kc, depending upon the species and the individual, but overtones and harmonics are present to a considerable degree (Novick, 1958). *Saccopteryx* and *Taphozous* (Emballonuridae) and some species of *Tadarida* (Molossidae) emit partly audible orientation cries. *Rhinopoma* also emit orientation pulses with audible components (Moehres and Kulzer, 1956a). *Rousettus, Steatornis,* and *Collocalia,* though, unlike all of the Microchiroptera, produce clicks with relatively little energy above 20 kc. Thus, it appeared that only relatively long wave-lengths would be available for echolocation and that *Rousettus* and the two cave-dwelling birds might be unable to detect obstacles as small as the wires that had been used as standardized test objects for the Microchiroptera (Hahn, 1908; Griffin and Galambos, 1941; Griffin and Novick, 1955; and Grinnell and Griffin, 1958).

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A single male *Rousettus aegypticus,* captured in a dimly lighted cave at Eaux Chaudes, Katana, Kivu Province, Belgian Congo in July, 1956, was brought to Harvard University in good health in August, 1956. This bat survived for nine months on a diet of bananas and, after a short period of recuperation from its journey and its restriction to a small cage, flew skillfully in an experimental flight room 32′ long, 12′ wide, and 8′ high. Its ability to avoid a variety of cylindrical test obstacles arranged in a row across the center of this room was tested by methods directly comparable with those previously used to measure obstacle-avoiding skill in the Microchiroptera. This *Rousettus* proved able to avoid surprisingly small wires even in total darkness. Its skill is here compared with that, measured previously, of the vespertilionid, *Myotis l. lucifugus* (Curtis, 1952).

This work was partly sponsored by the Office of Naval Research, the United States Public Health Service, and the Belgian American Education Foundation. During this time, Novick held a Post-doctoral Fellowship of the National Institute of Neurological Diseases and Blindness. We are grateful to the personnel of the *Institut pour la Recherche Scientifique en Afrique Centrale,* Lwiro, Belgian Congo for their help in capturing the experimental subject. Reproduction of this paper in whole or in part is permitted for any purpose of the United States government.

METHODS

After the bat had become accustomed to the problems of flight both in light and in total darkness in the flight room, and to the task of dodging between vertical obstacles suspended from the ceiling across the middle of the room, we tested its ability to avoid cylindrical obstacles, spaced 53 cm. apart, varying in size from cardboard tubes 5 cm. in diameter to bare metal wires 0.28 mm. in diameter. In each case these obstacles were suspended in a movable frame in a plane parallel to the end walls of the room. This plane had to be crossed by the bat in flying from its roost at one end to its roost at the other end. We forced such flights by agitating the roost which was a loosely suspended horizontal bar of wood. The bat would take off and fly the length of the room to the opposite roost or would, on occasion, make several flights back and forth before landing. In each of the tests considered below, the frame holding the obstacles was shifted horizontally in the dark just before each flight so that the absolute position of the obstacles and their location relative to the walls were unknown to the bat, though their position relative to one another was constant. Thus, the bat could not learn the location of the open spaces nor could it depend on following the walls because the space adjacent to the walls was frequently and randomly too narrow to permit passage. The room was totally dark during all these observations, but we often noticed by listening to the bat's audible clicks or to its wingbeats that it hesitated in front of the obstacles and executed dodging maneuvers to pass between them.

The *Rousettus* was thus required to fly through an obstacle plane and its accuracy of echolocation was evaluated in terms of its ability to avoid the obstacles. One must consider whether it was constantly and equally motivated to avoid collisions and whether its physical agility was sufficient for it to make the maximum use of its orientation system. The flights were scored simply as hits or misses by means of the sound of hits or in doubtful cases by inspecting the obstacles in light switched on immediately after the bat's passage. A hit always caused a clearly visible, sustained vibration of the obstacles as they were suspended from rubber bands. All hits were considered equal although some undoubtedly represented the bat's inability to maneuver successfully even though it had detected the obstacle, and some represented light touches by the wingtips which may have been sufficiently painless to call for no great effort to avoid their occurrence. Unlike the Microchiroptera, this *Rousettus* rarely turned back from the obstacles. Its position and attitude in passing through the obstacle plane were recorded on about 40 flights with a camera and electronic flash. All wing positions from completely spread to considerably folded were photographed both just before and just after passage through the barrier, but we could not determine whether the bat was reducing its potential collision diameter just at the obstacle plane. Its maximum wingspread was about 75 cm., and while we cannot accurately estimate its mean wingspread this must have been at least 45 cm. or very little below the spacing between the wires.

TABLE I

Comparison of the obstacle avoidance scores of a Rousettus aegypticus with those of Myotis l. lucifugus (Curtis, 1952). The wires or other cylindrical obstacles were arranged vertically and spaced 53 cm. apart for Rousettus and 30 cm. apart for Myotis

Diameter of obstacle (mm.)		*Myotis l. lucifugus*		*Rousettus aegypticus*	
		No. trials	% misses	No. trials	% misses
Cardboard tubes	25	—	—	109	76%
Rubber tubing	19	—	—	161	78%
Rubber tubing	13	—	—	100	77%
Rubber tubing	6	—	—	50	80%
Metal rods	4.76	140	85%	—	—
Insulated metal wires	3	—	—	442	85%
Bare metal wire	1.5	—	—	200	77%
Bare metal wire	1.21	3820	82%	—	—
Bare metal wire	1.07	—	—	280	68%
Bare metal wire	0.68	480	77%	—	—
Bare metal wire	0.65	—	—	225	58%
Bare metal wire	0.46	—	—	134	45%
Bare metal wire	0.35	660	72%	—	—
Bare metal wire	0.28	—	—	50	18%
Bare metal wire	0.26	600	52%	—	—
Bare metal wire	0.12	530	38%	—	—
Bare metal wire	0.07	460	36%	—	—

Finally, the possibility that the bat would detect the presence of the obstacles by their fastenings to the ceiling and/or floor and learn that they were suspended vertically between these two points was excluded by framing the obstacle plane with uniform fiberboard so that only the obstacles themselves and not their fastenings were exposed to acoustic or visual inspection. As a last precaution, lest the bat learn to recognize the position of the obstacles by listening to the movement of the frame between flights, the readjustment was covered with loud noise. The nature and size of the obstacles used are shown in Table I.

TABLE II

Experiments with a captive Rousettus exposed to thermal noise while flying through a row of vertical wires, 3 mm. in diameter spaced 53 cm. apart. All flights in total darkness except as noted. The noise was filtered with high pass (HP) or low pass (LP) electronic filters as noted

Date	Conditions of test	No. misses/No. trials	Per cent misses	Remarks
Apr. 23	Quiet	30/40	75%	
	Noise, 25 kc HP	0/10	0	Totally disoriented
	Quiet	17/20	85%	
	Noise, 25 kc HP with lights on	9/10	90%	Flew normally
	Noise, 15 kc LP	4/10	40%	Somewhat disoriented
	Quiet	7/10	70%	
	Noise, 15 kc LP	3/10	30%	Disoriented, but less so than at 25 kc HP
	Quiet	10/10	100%	
Apr. 26	Quiet	10/10	100%	Reluctant to fly
	Noise, 25 kc HP	0/8	0	Badly disoriented
	Quiet	6/6	100%	Very tired
Apr. 28	Quiet	8/10	80%	
	Noise, 15 kc LP	1/10	10%	Badly disoriented
	Quiet	4/10	40%	Tired
May 3	Died			
Averages of all tests	Quiet	93/116	79%	
	Noise, 25 kc HP	0/18	0	
	Noise, 25 kc HP with lights on	9/10	90%	
	Noise, 15 kc LP	8/30	27%	

RESULTS

The results are presented in tabular form. The only data excluded from consideration are those which were obtained when the bat was clearly fatigued or in poor condition near the end of a long series of trials or after many days of inactivity. The data are compared directly in Table I with similar data obtained by Curtis (1952) with *Myotis l. lucifugus*.

A short series of experiments was carried out to compare the resistance of *Rousettus* to interference with its echolocation by thermal noise but before further studies could be completed the bat died, possibly of injuries sustained in these experimental flights. The data are shown in Table II, because they indicate a markedly greater vulnerability to interference by noise than occurs with the Vespertilionidae (Griffin, 1958). Thermal noise was generated in 20 electrostatic loudspeakers adjacent to the plane of obstacles. This noise was limited in frequency band, by electronic filters, in one of two ways. Either the filter was set at 15 kc high pass so that frequencies above 15 kc were generated at a high level while lower frequencies were attenuated progressively at 24 db per octave, or else a 25 kc low pass filter was used to transmit audio frequencies while attenuating ultrasonic components of the noise, also at 24 db per octave. Without noise, the bat avoided 3 mm. wires 79% of the time in the dark. In the light, and with the noise, in a very short series, it avoided the wires 90% of the time. But in the dark the bat was incapable of avoiding these wires at all in intense noise above 25 kc. In noise below 15 kc, it scored 27% misses. The bat's total inability to avoid large wires in noise above 25 kc and its very poor performance in noise below 15 kc suggest several hypotheses. If we assume that the poor performance was due to unfavorable signal-noise ratio at the same frequencies, then we have evidence that *Rousettus* depends upon a wide range (from less than 15 kc to more than 25 kc) of frequencies in echolocation. But alternatively the analytical ability of *Rousettus*' ears may not suffice for distinguishing a 14 kc echo from either type of noise tested, that is, we may simply have shown that the accuracy of acoustic orientation in *Rousettus* can be reduced (even totally) by noise. The results may also have been complicated by the bat's panic, discomfort, loss of motivation, or confusion in an unusual situation aside from its ability to perceive echoes in a noisy environment.

Discussion

In these experiments, the wires were less widely spaced relative to the wingspread of *Rousettus* than in Curtis' experiments with *Myotis*, but *Rousettus* almost always approached the plane of the obstacles perpendicularly while *Myotis* often approached obliquely. Our flight room was also considerably larger than the 15′ × 9′ × 6′ room used by Curtis. The percentage of misses for relatively large obstacles was, nevertheless, almost exactly the same—85.0% for *Myotis* with 4.76-mm. rods and 84.5% for *Rousettus* dodging 3-mm. wires. *Rousettus* was slightly less successful at avoiding even larger obstacles (cardboard and rubber tubes) but these tests were conducted early in the bat's experience in the exacting task of flying in a dark room (with its multiplicity of echoing surfaces).

This *Rousettus* was able to detect and avoid, with a considerable degree of success, wires as small as 1.07 mm. in diameter. Only when confronted with wires of less than 1 mm. did its skill fall seriously below its own standards as well as those of *Myotis*. *Rousettus*' score decreased rather gradually. If we consider its poor performance (18% misses) against 0.28-mm. wires as due to chance, then *Rousettus* was clearly detecting 0.46-mm. wires against which it scored 45% misses. Even 18% misses against 0.28-mm. wires may have represented some degree of echolocation for, when flying in a noise field, this bat did even more poorly (100% hits) against 3-mm. wires. It seems reasonable that the ease with which a small object is echolocated depends upon its position relative to the angle of sound emission and its beaming and the angle of sound reception. Thus there is likely to be an optimal angle of approach (probably, but not necessarily, straight ahead) where the maximum echo will be received and less easily detected obstacles will be echolocated. Obstacles which lie less optimally relative to the bat will have to have more effectively echoing surfaces to be detectable. Thus the bat might well succeed in avoiding a 0.46-mm. or 0.65-mm. wire only if it chanced to approach it favorably and so its score when working against obstacles of marginal size would be an average of chance misses, active misses, and "blind" hits. One of the limiting factors in exploring the threshold of echolocation is the danger of serious injury to the bat every time it collides with an obstacle. Such collisions may be major accidents or simply touches. Collisions with small wires tend to be more serious than those with large obstacles. *Rousettus'* performance varied considerably from trial to trial. Whenever possible we ran long series of tests and interspersed tests with 3-mm. wire between those with smaller sizes. The results were consistent with the average scores listed in Table 1.

The design of *Myotis* orientation pulses is very different from that of *Rousettus* clicks. *Myotis* pulses are produced laryngeally and emitted through the open mouth. They have a frequency modulated pattern with a gradually falling frequency starting on the average at about 80 kc and ending at about 40 kc but with beginnings ranging from at least 60 to 120 kc. Similar variety among terminal frequencies also occurs. Thus *Myotis* in single pulses and in consecutive pulses produce prominent frequencies covering about two octaves (Griffin, 1958; Novick, 1955). Furthermore, harmonics also occur in *Myotis* pulses and represent a second octave sweep within the pulses in which they occur. The importance of the harmonics as components of the outgoing pulses and the returning echoes and in the carrying of information about the environment to the bats has not been evaluated. In *Rousettus*, the pulses are produced by tongue clicks and are impure in frequency and irregular in frequency pattern. The bulk of the energy, however, appears to be in the range of about 12 to 18 kc. Additional energy is scattered from 6.5 to over 100 kc with a second maximum at about 20 to 40 kc (Moehres and Kulzer, 1956a; Kulzer, 1956; Novick, 1958).

SUMMARY

1. The ability of a single specimen of the fruit bat, *Rousettus aegypticus*, to avoid test obstacles of various sizes by echolocation in total darkness was tested. This bat avoided vertically placed 3-mm. metallic wires 85% of the time. Its success declined gradually as the wires were reduced in size but the bat displayed considerable success (68% misses) against 1.07-mm. wire and did significantly better than chance (45% misses) against wires 0.46 mm. in diameter.

2. These results have been compared with those of Curtis (1952) who studied the vespertilionid, *Myotis l. lucifugus*.

3. *Rousettus'* success at echolocation was considerably reduced when it was forced to fly in a field of intense thermal noise.

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ELECTROPHYSIOLOGICAL STUDIES OF ARTHROPOD CHEMORECEPTION. III. CHEMORECEPTORS OF TERRESTRIAL AND FRESH-WATER ARTHROPODS[1]

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While an extensive literature documents the role of chemoreceptors in the behavior of invertebrates (Hodgson, 1955), the small size of chemoreceptor cells is a major handicap in any attempt to study their functions using conventional electrophysiological procedures (Chapman and Craig, 1953; Roys, 1954). Barber (1956) recorded afferent impulses from neurons which supply the gnathobase chemoreceptors of *Limulus* and noted an increase in nerve activity when aqueous extracts of marine bivalves were applied to the gnathobase. Use of microelectrodes enabled Schneider (1957) to record afferent impulses from groups of antennal chemoreceptors in male silkmoths (*Bombyx*) during stimulation with extracts of the scent glands from female moths. Possible synaptic effects between receptor cells and nerves supplying them, or the unpredictable numbers of cells represented in most recordings, make it difficult, however, to interpret the results in terms of single unit activity of the actual chemoreceptor cells.

A relatively simpler technique is that of recording the afferent impulses from primary chemoreceptor cells through the same fluid which is applied as a stimulus (Hodgson, Lettvin and Roeder, 1955). This method has thus far been applied only in studying contact chemoreceptors of two animals: labellar chemoreceptors of the blowfly *Phormia* (Hodgson and Roeder, 1956; Wolbarsht, 1957) and tarsal chemoreceptors of the butterfly *Vanessa* (Morita *et al.*, 1957). The conclusions from studies of these two preparations point to a number of unexpected properties of primary chemoreceptor cells.

With both *Phormia* and *Vanessa*, it was found that different chemoreceptor cells were specialized to respond, not to the different modalities of stimuli generally held to be effective for contact chemoreceptors of vertebrates (*e.g.* Beidler, 1952), but either to sugars or to various non-sugars, with the presence of a water-specific receptor also strongly indicated in *Vanessa* (Morita *et al.*, 1957). Seemingly at variance with the usual concept of single specificities of receptor cells (Granit, 1955), a single primary receptor cell of *Phormia* may respond to chemical, tactile, and thermal stimuli within normal physiological ranges (Hodgson and Roeder, 1956). Unfortunately, information on this point is not available for *Vanessa*.

In view of these unexpected results, and the lack of any comparable electrophysiological data on primary chemoreceptors of other invertebrates, it seemed desirable that the method of recording through fluid-filled, externally applied electrodes

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[1] This investigation was aided by Public Health Service Grant No. E-1010, and by the Higgins Fund of Columbia University. The field work was aided by a National Science Foundation Grant to the Mountain Lake Biological Station.

should be tried on chemoreceptors of a wider variety of animals, in order to determine how generally the characteristics found in *Phormia* and *Vanessa* receptors may apply to the functions of other primary chemoreceptor cells. For technical reasons, this method is best adapted to recording from chemoreceptors in arthropods (Hodgson, Lettvin and Roeder, 1955). The object of the present paper is to report the results of tests conducted using this method upon the chemoreceptors of some terrestrial and fresh-water arthropods. In each case where the method could be successfully applied, answers to the following questions were sought: (1) Does the same receptor cell respond to chemical, tactile, and thermal stimuli within normal physiological ranges? (2) What modalities of chemical stimuli excite the individual primary chemoreceptor cells? (3) Does the relationship between the reaction of the animal to chemicals and the range of sensitivity of its chemoreceptors indicate a peripheral discrimination mechanism, such as found in *Phormia*?

Methods

Thirty-seven species, representing the major classes of arthropods and eight orders of insects, were tested. These species are arranged according to taxonomic status below. All specimens were collected in the field and tested within 12 hours after capture. The animals were allowed to drink water to repletion, but no attempt was made to control their diet prior to testing. At least three individuals, usually more, belonging to each species were studied.

The technique of recording action potentials from chemoreceptors using externally applied, fluid-filled electrodes has been described in detail elsewhere (Hodgson, Lettvin and Roeder, 1955; Hodgson and Roeder, 1956). This technique was used with only such minor modifications as were necessary to manipulate the variety of receptor-bearing appendages tested. All experiments were tape recorded and photographs made from the tape recordings, beginning one-half second after the stimulus was applied, thus avoiding the base-line fluctuations which commonly accompany the stimulus artifact.

The species tested were as follows, with each group and each species yielding potentials from chemoreceptors designated by an asterisk. (Except as otherwise noted, identifications were checked through the courtesy of Dr. R. E. Blackwelder of the U. S. National Museum.) *Class:* Crustacea*—Cambarus bartonii sciotensis* (Det. H. H. Hobbs, Jr.); *Class: Arachnida*—Latrodectus mactans (black widow spider), Theridion tepidariorum (house spider); *Class: Diplopoda**—Pseudotremis sp. (Det. H. F. Loomis), Pseudopolydesmus serratus* (Det. M. Walton); *Class: Insecta; Order: Odonata*—Aeschna constricta, Libellula pulchella, Progomphus obscurus; *Order: Orthoptera**—Acheta assimilis (common field cricket), Ceuthophilus gracilipes* (cave cricket), Crytocercus punctulatus (wood-eating roach) (Det. L. R. Cleveland), Hadenoecus putaneus* (cave cricket), Scudderia furcata (katydid); *Order: Hemiptera*—Arilus cristatus, Oncopeltus fasciatus (large milkweed bug); *Order: Coleoptera*—Cicindela sexguttata (six-spotted tiger beetle), Dineutes americanus (whirligig beetle), Dytiscus fasciventris (large diving beetle), Laccophilus maculosus (common pond beetle), Nicrophorus tomentosus (carrion-beetle), Phymatodes dimidiatus (longhorn beetle), Saperda candida (apple tree borer), Silpha americana (carrion beetle), Tropisternus lateralis (keeled water beetle); *Order: Megaloptera*—Carydalus cornutus (dobsonfly); *Order: Neurop-*

tera—Chrysopa sp. (golden eyed lacewing); *Order: Diptera**—Amoebaleria defessa* (cave fly) (Det. C. H. Curron), Tipula trivittata (crane fly); *Order: Lepidoptera**—Atlides halesus (purple hairstreak); Epargyreus clarus* (silver spotted skipper), Limenitis arthemis astyanax* (red spotted purple), Papilio marcellus* (zebra swallowtail), Papilio philenor* (pipe vine swallowtail), Protoparce quinquemaculata (five-spotted hawk moth), Speyeria cybele* (great spangled britillary), Tropaea luna (luna moth), Vanessa atalanta* (red admiral).

The chemicals tested were sodium chloride, sucrose, d-levulose, glycine, DL glutamic acid, citric acid, oil of citronella and oil of wintergreen. Sodium chloride was tested as a 0.25 molar aqueous solution. Oils of citronella and wintergreen were tested by bringing swabs soaked in these chemicals to within an inch of the sensory structure. Although quantitative control of stimulus concentration was not obtained by this method, the results obtained with these two oils were quite reproducible. All of the other chemicals were mixed with sodium chloride so that the final test solution was an unbuffered aqueous solution containing 0.1 molar NaCl and a 0.25 molar concentration of the test chemical. Results were compared with activity recorded when 0.1 molar NaCl was applied alone.

Temperatures were measured with a thermistor implanted just under the cuticle near the receptor being studied. The temperature was changed by bringing a warm glass rod or small ice-pack near the preparation. Spike potentials from mechanoreceptors were recorded by bending sensilla or whole appendages with needles. Certain departures from the usual tests are described at appropriate points below.

RESULTS

All of the preparations yielded numerous spike potentials originating from tactile receptors, thus providing assurance that the preparations were alive when studied. In only five orders of the arthropods tested, however, was it possible to obtain unequivocal recordings from chemoreceptors. These five groups are designated by asterisks above. The several factors believed to be responsible for failure to record action potentials in all of the tested species are considered in the discussion, and a complete description of the results will be presented only for those forms in which chemoreceptors could be studied using fluid-filled electrodes.

1. DECAPODA *Cambarus bartonii sciotensis* (16 individuals)

This large crayfish proved to be an exceptionally interesting experimental animal. Recordings could be made with the usual 0.1 molar NaCl conducting solution in the electrode, or else by using distilled water or pond water as a solvent for the chemicals. Although the results showed few differences whichever solvent was used, all of the tests were run with chemicals dissolved in distilled water, thus avoiding any possible complications of the sodium chloride.

The antennae and the lateral branches of the antennules were alike in yielding only records of mechanoreceptors at low amplitudes (30 μV). From the entire medial branch of the antennule, however, it was possible to record a variety of spike potentials ranging in amplitude from 30 μV to 500 μV. The large-amplitude spikes (200 μV to 500 μV) were recorded only when the antennule was bent. Consequently, the cells giving rise to these potentials, which are relatively few in this

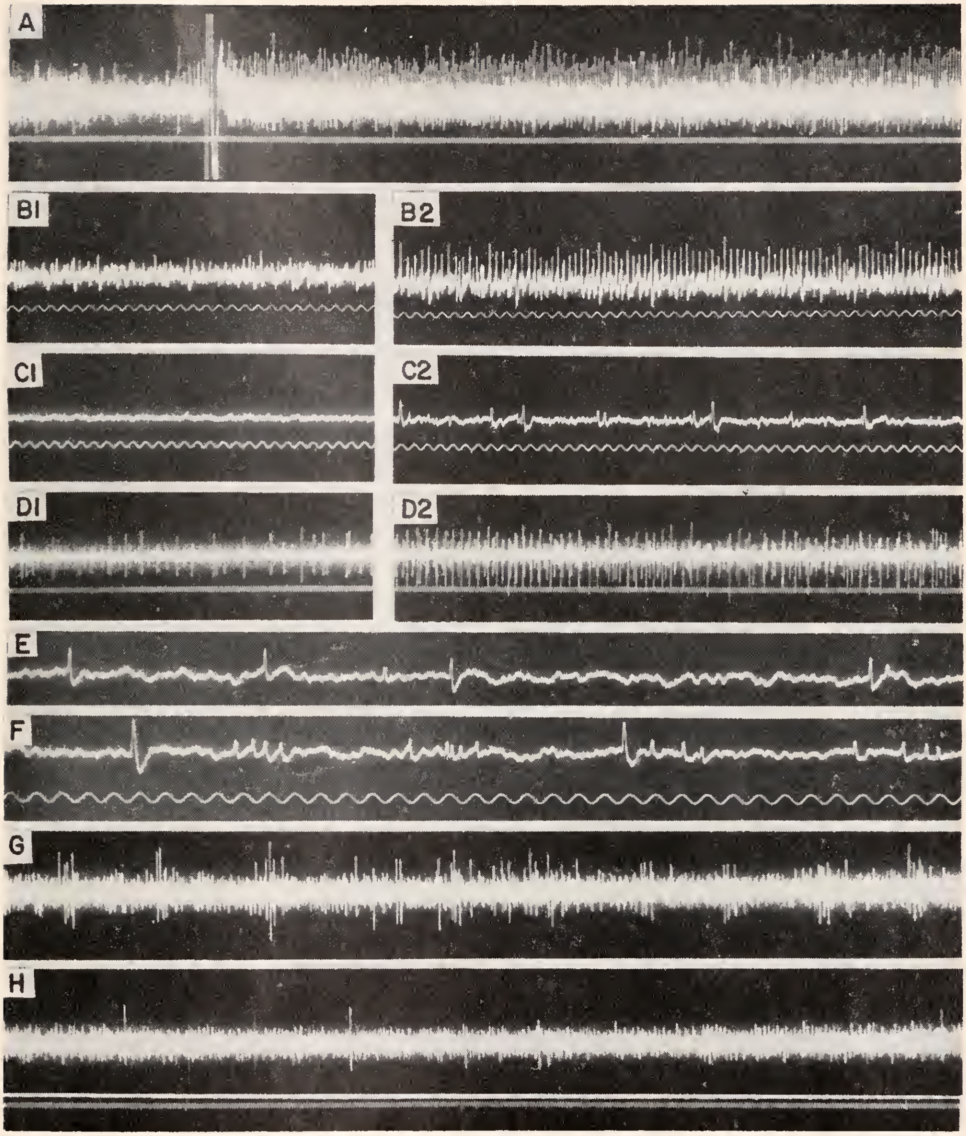

FIGURE 1. Typical spike potentials from arthropod chemoreceptors. *A*, response of medial branch of *Cambarus* antennule to glutamic acid; *B1*, single sensillum on *Cambarus* walking leg, tested with distilled water; *B2*, same as *B1*, except glycine test solution; *C1*, *Pseudopolydesmus* tarsus, NaCl control; *C2*, same as *C1*, except sucrose test solution; *D1*, spontaneous activity, *Hadenoecus* tibia; *D2*, same as *D1*, except exposed to citronella vapor; *E*, single tarsal sensillum of *Epargyreus*, control NaCl solution; *F*, same as *E*, except sucrose test solution; *G*, antenna of *Amoebaleria*, distilled water in electrode; *H*, same as *G*, except exposure of antenna to oil of citronella vapor. Time bases for all records, 100 cycles per second. Consult text for additional details.

branch of the antennule, are mechanoreceptors. The majority of the spikes have amplitudes of 30 μV to 50 μV. These respond to the application of glycine and glutamic acid, of the test series of chemicals used. Because a number of different amplitudes of spikes were recorded even with the smallest practicable areas of electrode contact, it was not possible to determine whether identical cells were responding to both chemical and tactile stimuli. Record A of Figure 1 is taken from an experiment in which a test solution of glutamic acid was allowed to flow around the medial branch of an antennule without changing electrode contact. Activity recorded when the antennule is in distilled water (on the left of the large stimulus artifact) is negligible, but many small-amplitude spike potentials follow the introduction of the glutamic acid. The frequency of firing during chemical stimulation was not influenced by temperature changes within the range tested—five degrees (C.) above or below the room temperature of 25 degrees.

Chemoreceptors were also found on the first two pairs of walking legs. The chemoreceptors were located on the chelae and, to a lesser extent, elsewhere on the protopodites of those legs. The external chemosensory structures can be recognized in *C. bartonii* as tufts of setae, numbering ten to twenty setae per tuft, each such tuft arising from a circular depression in the cuticle. Contact of the electrodes with other parts of the cuticle failed to terminate the open circuit condition between the indifferent electrode inside and the recording electrode outside the cuticle. The best records were obtained after the claw had been allowed to dry at room temperature for thirty minutes following its removal. This prevented short circuits between the recording and indifferent electrodes. By teasing apart the hairs of a single tuft, the tip of an electrode could then be positioned over a single sensory hair. In this way the firing of a single chemoreceptor cell could be studied. The spike potentials recorded from different sensilla ranged from 30 to 60 μV in amplitude. It was found that these receptors resemble chemoreceptors on the antennule in being insensitive to test chemicals other than amino acids of the series used. (Records B1 and B2 of Figure 1 illustrate typical results during applications of a control NaCl solution, and the test mixture of NaCl and glycine, respectively.) The chemoreceptors on the first two walking legs were never observed to respond to mechanical movement of the sensory hairs during recordings. The small size of the hairs (about 20 microns in length) and their position surrounded by rigid cuticle would appear to render their usefulness as tactile receptors unlikely. The insensitivity of these receptors to temperature changes within the range tested resembles that of the receptors on the antennule. Impulses from chemoreceptors were not detected from the chelipeds, third maxillae, or elsewhere on the body of the crayfish using the present method.

Behavioral experiments were run to check the possibility of a peripheral discrimination for amino acids. Ablations of antennae, antennules, or the first two pairs of walking legs, and combinations of these operations, were performed on thirty crayfish. The results were difficult to interpret in many cases because of abnormal behavior of operated animals. It was easy to demonstate, however, that the animals can locate food using the first two pairs of walking legs, even when antennae, antennules, and maxillipeds are removed. Activity resembles that during normal feeding and can be initiated by injecting 0.25 molar solutions of glycine and glutamic acid into the water, while even intact animals fail to give clear-cut responses to the other test solutions. Thus there seems to be a clear correlation between the electrophysiological data and the behavioral results. Attempts to determine by behavioral tests whether the antennae and lateral branches of the antennules bear chemoreceptors yielded results which could not be unequivocally interpreted. Doflein (1910), on the basis of behavioral tests, has reported that the antennules of decapods contained chemoreceptors, and Luther (1930), using similar methods, reported chemoreceptors on mouth parts, walking legs, and pincers of brachyurans.

2. DIPLOPODA *Pseudotremis* sp. (4 individuals); *Pseudopolydesmus serratus* (4 individuals)

In both *Pseudotremis* and *Pseudopolydesmus* many action potentials could be recorded from the tips of the antennae and from the tips of the legs when an electrode filled with 0.1 molar NaCl was applied to those parts. In *Pseudotremis*, the smaller species, the action potentials were never more than 40 μV in amplitude, and all clearly responded to mechanical bending of the antenna or leg. In *Pseudopolydesmus* the largest spikes from the antenna were about 60 μV in amplitude, and those from the tarsus were about 80 μV. All of the larger spikes increased in frequency during bending of the appendages being tested, and it was therefore assumed that these spikes represented the afferent impulses from mechanoreceptors. Spike potentials of smaller amplitude (30–50 μV) from tarsi of *Pseudopolydesmus* occurred with increased frequency when the tarsi were bent, or sugars applied. (See Fig. 1C.) They did not change during application of other test solutions or during temperature changes within five degrees (C.) of the room temperature of 25 degrees. No significant changes in the frequency or pattern of impulses were noted in recordings from the antennae of the two species when chemical stimuli were applied.

The small trichoid sensilla which probably enclose the actual chemosensory cells on the tarsi of *Pseudopolydesmus* are too closely spaced to make possible a restriction of the area of electrode contact to a single sensillum. Attempts to record activity using electrodes filled with distilled water were likewise unsuccessful. In view of the smaller size of the mechanoreceptor spikes recorded from *Pseudotremis*, and the generally smaller size of action potentials from chemoreceptors as compared with mechanoreceptors, it would hardly be expected that chemoreceptor spikes from *Pseudotremis* would be detectable above the inherent "noise level" of the apparatus. Behavioral test showed that sucrose or levulose, placed in contact with the tarsi, initiated feeding responses even after the antennae were removed. Tarsal contact with citric acid caused the animals to move away from the test solution, but this was the only test solution, other than the sugars, which elicited a behavioral response. With the exception of citric acid, receptors for which could not be detected electrophysiologically, the behavioral and electrophysiological results suggest the existence of a peripheral discrimination mechanism.

3. ORTHOPTERA *Ceuthophilus gracilipes* (7 individuals); *Hadenoecus putaneus* (3 individuals)

The orthopterans tested showed considerable variation, some of which appears to be related to habitat. *Cryptocercus*, a wood-eating roach, was completely refractory to the recording method, except for a few mechanoreceptors in the antennae and palpi. A larger number of tactile receptors were recorded from the antennae and palpi of the katydid, *Scudderia*, and the field cricket, *Acheta*. Best results, however, were obtained with the cave crickets *Ceuthophilus* and *Hadenoecus*, which have antennae elongated to many times the length of the body and also have unusually long legs and palpi. The data support the generally expressed assumption that these anatomical modifications are associated with hypertrophy of tactile and chemical senses which would presumably be of selective value in dark subterranean environments.

In tests of seven adult specimens of *Ceuthophilus* and three of *Hadenoecus*, the antennae were found to contain spontaneously active and quick-adapting mechanoreceptors (spike amplitudes 50–80 μV) along with spontaneously active, relatively non-adapting chemoreceptors (spike amplitude 20–40 μV). The latter were seen in one antennal preparation of *Ceuthophilus* and all three preparations of *Hadenoecus*. The frequency of the small spikes did not change during application of any of the test chemicals in solution, or during temperature changes between 20 and 30 degrees C., but did increase when swabs soaked in citronella or wintergreen were brought near the region of the antenna in contact with the electrode. Essentially similar results were obtained from recordings of the receptor activity in both the maxillary and labial palpi and the tarsi of *Ceuthophilus* and *Hadenoecus*. In addition, small spikes (30–50 μV) were recorded from the trochanter and tibia of the prothoracic and mesothoracic legs of *Hadenoecus*, in six out of eight preparations when the legs were exposed to vapors of wintergreen or citronella. Mechanical bending of sensilla on the trochanter and tibia also increased the frequency of these same spike potentials. Record D1 of Figure 1 shows the spontaneous activity of receptors in the tibia of a prothoracic leg of *Hadenoecus*, and record D2 shows the increase in frequency of spikes during application of citronella vapor. No effects of the test chemicals in solution could be detected in either *Ceuthophilus* or *Hadenoecus*, and chemoreceptor activity could not be recorded from the cerci, ovipositor, general body surface, or the larger spines on the legs of either species. *Ceuthophilus* did not give any clear-cut behavioral response to citronella or wintergreen in tests of the intact animals, but *Hadenoecus* gave intense avoidance reactions, moving quickly away from these stimuli. Removal of the antennae and palpi did not abolish this reaction in *Hadenoecus*, which always responded most strongly when stimuli were near the legs.

4. LEPIDOPTERA

Nine species of Lepidoptera were tested. Only a few impulses associated with tactile stimulation could be recorded from the antennae of any of these species, even when vapors were applied. In all six species of butterflies tested, records were obtained from the tarsal receptors (described by Minnich, 1921). Tests upon the tarsal sensilla of *Epargyreus* and *Limenitis* revealed that each sensillum had a few receptor systems functioning similarly to that in the labellar hairs of *Phormia*. (Compare the records E and F of Figure 1, taken from tests of a single tarsal sensillum of *Epargyreus*, and note that the small spike potentials predominate only in record F when sugar is present in the electrode.) The maximum number of receptors represented in recordings from single sensilla of these two species is four, and the minimum two. Variations within these limits were commonly encountered in comparisons of the records from several hairs, even on the same tarsus. The variations characteristically occurred in the smaller spike potentials, but under the conditions of these tests all of the smaller spikes increased in frequency during stimulation with sugars, and the largest spike responded with increased frequencies during application of any of the non-sugar solutions. These receptors were not observed to respond to vapors of citronella or wintergreen.

With the other species of butterflies tested, there appeared to be as many as 12 different receptors associated with each tarsal sensillum and the records were too complex for analysis of the functions of any single receptor cells. Responses to tactile stimulation were obtained in tests with tarsal hairs of all the butterflies used; in those preparations involving only a few fibers it was clear that all fibers responded to bending of the tarsal hair, and probably this was the case with the many-fiber preparations also, but this could not be determined with certainty because of the complexity of the records. The frequency of impulses recorded during continuous stimulation of single sensory hairs of *Epargyreus* and *Limenitis* was increased by temperature rises of as little as 1.2 degrees C. These particular tarsal receptors, then, bear a greater resemblance to the labellar chemoreceptors of flies than do any of the other preparations (excluding the labellar chemoreceptors of *Amoebaleria*) encountered in this survey. Feeding responses (proboscis extensions) in butterflies are known to be elicited by sugars, with negative responses being elicited by other types of chemicals (Dethier, 1953; Minnich, 1921). A peripheral mechanism for discrimination of acceptable and unacceptable chemicals is thus indicated by both the behavioral and electrophysiological results with butterflies.

Tarsal chemoreceptors were not detected in any of the three species of moths. No impulses could be recorded from the trichoid sensilla described by Frings and Frings (1949) on the proboscis of lepidopterans. The characteristics of the records obtained from such tests indicated, however, that a short-circuit between the recording and indifferent electrodes, established through the fluids in the proboscis, probably accounted for the lack of any spike potentials detected through an active electrode near the tip of the proboscis.

5. DIPTERA *Amoebaleria defessa* (7 individuals); *Tipula trivittata* (3 individuals)

Studies on four genera of Diptera having been previously reported (Hodgson and Roeder, 1956), the present work was confined to two types in which the chemoreceptors might be expected to be of special interest. The helomyzid fly *Amoebaleria* was tested because of its occurrence in caves, a habitat often associated with hypertrophy of chemical or tactile senses (Hodgson, 1955), and the crane fly *Tipula* was tested because the branching structure of its antennae suggested that recordings might be made from one or a few antennal receptors in a single antennal branch. Only *Amoebaleria* yielded results of interest, however.

The labellar chemoreceptors and chemoreceptors within the tarsal hairs of *Amoebaleria* proved to function similarly to those in *Phormia*, in that they exhibited *L* and *S* spikes when stimulated by sugars or non-sugars, and showed comparable responses to tactile and temperature stimulation. Some data on olfactory receptors were obtained in recordings from the antennae of *Amoebaleria*. A typical result, obtained by placing a fluid-filled electrode on the antenna, is shown in record G of Figure 1. Distilled water is adequate in the electrode, and the results are essentially the same whether contact is made with the distal tip of the antenna or the enlarged third segment near the base of the antenna. Ablation experiments show that most of the activity recorded originates in the third segment of the antenna in either case. The abundant spikes which seem to represent the basal level of receptor activity in the absence of externally applied stimulation are not affected by any of the test solutions applied, but are decreased in frequency by vapors of wintergreen, or citronella (see record 11 of Figure 1). This result was so contrary to anticipated findings that tests were run with benzene, toluene, and carbon tetrachloride vapors, all of which produced similar reversible decreases in amount of receptor activity. Unfortunately, so little is known of the natural history of this fly that it is impossible to say what might constitute the normal olfactory stimuli.

Tactile effects upon the antennal receptors were observed only when the surface of the antenna was prodded or bent in excess of any amount of stimulation which the antenna would encounter in flight. Blowing upon the antenna during a recording or varying the temperature from 20 to 28 degrees C. produced no discernible effect upon the frequency or pattern of the impulses recorded. Attempts to make similar antennal recordings using other species of flies have yielded only negative results.

Discussion

In view of the considerable differences in chemoreceptors which have already been reported from electrophysiological studies of mammals (Beidler, Fishman and Hardiman, 1955) it is not surprising that much greater differences should be found among members of such a heterogeneous group as the arthropods. It seems clear that sensitivities to tactile and temperature stimuli within the normal physiological range are not essential characteristics of primary chemoreceptor cells, even among the arthropods, because several exceptions to this situation were found as soon as tests were made of chemoreceptors other than those on the fly labellum. Yet it would probably be incorrect to regard the labellar receptors as primitive or unspecialized receptor cells. Their similarity to receptors in the tarsal sensilla of at least two of the butterflies tested suggests that a sensitivity of the same cell to more than one type of energy in the environment may have a high selective value in cases where only a relatively small number of receptors contact a substrate, many features of which are significant for the animal's behavior. This certainly would be the case with receptors on the tarsus or proboscis of a fly or butterfly, or on the tips of the tarsi of a millipede. The demonstrated multiple sensitivities of single receptor cells in those locations may, therefore, be one of the solutions which evolution has produced for the problem of obtaining a variety of information about the environment when only very small areas of the body are actually in contact with the environment. Whether the several types of stimuli all eventually affect the same excitatory process within a single receptor cell will have to be determined by further investigations. Cases of double specificities of receptors in vertebrates, such as the temperature-touch receptors of the rattlesnake facial pit (Bullock and Diecke, 1956), have been reported but it is very doubtful that more than one type of stimulation *normally* acts upon the same receptor units, and even if this were true these would have to be considered exceptions to the general rule of single specificities for single receptors (Granit, 1955).

Several correlations might be noted between receptor distribution or function and the natural history of the particular animals concerned. Of the two cave crickets providing favorable receptor preparations, *Hadenoecus*, with the more extensively distributed chemoreceptors on the legs, is reported to be more strictly limited to caves than *Ceuthophilus* (Giovannoli, 1933). The selective advantage of highly developed chemical senses in a totally dark environment is obvious. The sensitivity of the chemoreceptors of *Cambarus* to amino acids is undoubtedly related to a diet of decaying meat, and the absence of any response of its receptors to sugars can be correlated with the lack of any behavioral response to sugars by this species. The results with butterflies likewise indicate the existence of a peripheral discrimination mechanism for the chemicals constituting the normal food in this case, sugars.

All of the spike potentials recorded from chemoreceptors were smaller in amplitude than the spikes from mechanoreceptors of the same animal, unless the same receptor cell responded to both types of stimuli. This is in accord with the usual assumption that chemoreceptor fibers are smaller than mechanoreceptor fibers (Dethier, 1953; Hodgson, 1955). The fact that many receptors in *Cambarus*, *Hadenoecus*, and *Amoebaleria* showed spontaneous activity supports another idea believed to be of some general applicability—that spontaneous activity is widespread among sensory cells, and that *any changes* in the frequency or pattern of the spontaneous activity (rather than the mere presence of impulses) may constitute the afferent "message" from the sense organs (Roeder, 1955). The antennal receptors of *Amoebaleria*, showing decreased numbers of impulses during administration of vapors, may illustrate a less common direction of change in spontaneous activity which serves as the afferent message.

The present experiments resolve a discrepancy between the earlier work on the labellar chemoreceptors of the blowfly (Hodgson, Lettvin and Roeder, 1955) and the results obtained by Morita *et al.* (1957, and personal communication) using the butterfly, *Vanessa*. The polarity of the spike potentials recorded from *Phormia* was previously reported as negative, using the present recording method, but positive under similar conditions in *Vanessa*. All the spike potentials recorded from chemoreceptors in the present study resulted from an increase in positivity at the distal tip of the sensory hairs (position of the recording electrode) relative to the base of the same hairs (position of the indifferent electrode), and the contrary polarity reported in *Phormia* was subsequently traced to an error in instrumentation. A precise explanation for the positive spike potentials obtained by this method cannot be given at the present time, but might possibly be explained by generation of the main negative spike potential at the cell body region of the receptor, which would leave the actual chemosensory area with a relatively positive charge. Experiments to localize the main impulse generating area within the receptor are now underway.

The failure to record potentials from chemoreceptors in a large majority of the arthropods tested could result from a real absence of these receptors in the appendages tested or from limitations of the technique. The latter is the more probable explanation in most cases. Particularly unfortunate is the apparent inapplicability of the technique to recordings from the antennae of most insects. Unavoidable short circuits between indifferent and recording electrodes explain some negative results, as noted above, but inability to position the recording electrode over one or a few receptor sensilla and the small size of the spike potentials from the chemoreceptors undoubtedly account for most of the failures. The optimum preparation for use with this technique appears to be an elongated sensillum, well isolated from surrounding sensilla, and containing very few receptor cells—an ideal approached more conveniently in the labellar chemoreceptors of flies than with any other arthropod preparations yet tested. A similar survey of the chemoreceptors of marine arthropods is planned.

It is a pleasure to acknowledge the courtesy of Dr. Horton H. Hobbs, Jr., Director of the Mountain Lake Biological Station, who facilitated the field work in many ways. Mr. David Bardack assisted in collecting the animals. Drs. V. G. Dethier and K. D. Roeder have been most helpful in critically reading the manuscript.

SUMMARY

1. Electrophysiological tests with externally applied, fluid-filled electrodes were performed upon thirty-seven species representing four classes of arthropods. Afferent chemoreceptor impulses were recorded in animals of five types: a crayfish (*Cambarus*), a millipede (*Pseudopolydesmus*), two orthopterans (*Ceuthophilus* and *Hadenoecus*), a helomyzid fly (*Amoebaleria*), and six species of butterflies.

2. Receptors sensitive to chemical, tactile, and temperature stimuli within normal physiological ranges are found in certain Lepidoptera (*Epargyreus* and *Limenitis*) and Diptera (*Amoebaleria*). Receptors with a dual sensitivity to at least two of the above types of stimulation are found in *Pseudopolydesmus*, *Ceuthophilus*, and *Hadenoecus*. It is concluded that multiple sensitivities of receptors are not exceptional in arthropods.

3. Chemoreceptors sensitive to amino acids, but insensitive to tactile and temperature stimuli, are found on the chelae and protopodites of the first two walking legs of *Cambarus bartonii sciotensis*.

4. With the present recording method, spike potentials from chemoreceptors represent increases in positivity at the distal tip of the receptor cell, relative to the cell body.

5. Relationships between functional characteristics of chemoreceptors and the natural history of the animals are discussed.

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MORPHOLOGY OF MAIN AND ACCESSORY ELECTRIC ORGANS OF NARCINE BRASILIENSIS (OLFERS) AND SOME CORRELATIONS WITH THEIR ELECTRO-PHYSIOLOGICAL PROPERTIES

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Like other Torpedinidae (Bigelow and Schroeder, 1953), *Narcine brasiliensis* (Olfers) possesses electric organs. While they have previously been studied physiologically (Chagas *et al.*, 1953; Cox and Breder, 1943) no anatomical descriptions seem to have been published of the organs and their innervation, presumably because these have been considered to be identical with the findings in *Torpedo*. In the course of a new study (unpublished data from this laboratory) of the electrophysiology of *Narcine* electric organs with intracellular recording, certain discrepancies were observed which indicated differences between the structure of *Torpedo* organs and those of *Narcine*. A hitherto undescribed, smaller, accessory organ was also found, distinct in its electrophysiological properties from that which will now be called the main electric organ. Anatomical data to be reported here show that the accessory organ also differs from the major organ in structure, innervation, and in the size and orientation of the electroplaques. The present paper reports chiefly the gross anatomical and general histological findings, relating these to the functional properties of electric organs. Data on the embryology and on the fine structure of the electroplaques, including detailed studies of electron microscopic preparations, will be reported elsewhere (Mathewson and Lehrer, and Mathewson and Wachtel, unpublished data).

MATERIALS AND METHODS

Narcine brasiliensis inhabits the inshore waters of the Atlantic Ocean from Brazil to North Carolina (Bigelow and Schroeder, 1953). It is one of the smallest of the Torpedinidae, the 42 adult specimens brought into the Marineland Research Laboratory for the present study ranging from 20 to 45 cm. in length. In contrast, the larger of the *T. occidentalis* dissected by Hunter (*cf.* Keynes, 1956) measured about 1.25 m. Although it seems that specimens of this size have now become rare (Bigelow and Schroeder, 1953), a fish almost 0.75 m. long was brought into the Marine Biological Laboratory at Woods Hole in 1956 (Grundfest, 1957a).

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[3] This work was supported in part by grants from Muscular Dystrophy Associations of America, National Institutes of Health (B–389 C2), National Science Foundation, and United Cerebral Palsy Research Foundation.

[4] We express our thanks to the Trustees and staff of the Marineland Research Laboratory, where the initial phases of the work were carried out. To Mr. F. G. Wood, Jr., Curator of the Laboratory, we are especially indebted for his hospitality during our stay and for his continued cooperation.

Many of the fish available to us were used chiefly for electrophysiological work, and dissection at this time was confined to a minimum. The electrophysiological data provided clues to probable anatomical structures which were then sought for in other specimens, studied in fresh dissection, or in preserved preparations. Histological material was prepared with a number of techniques for different purposes. The details of these methods will be given in later publications.

RESULTS

A. The Main Organs

Gross anatomy. Superficially the paired, kidney-shaped main electric organs of *N. brasiliensis* (Fig. 1) resemble closely those of the other Torpedinidae (Bigelow and Schroeder, 1953; Fritsch, 1890). They comprise about twenty per cent of the total weight of the fish. Both dorsally and ventrally the main electric organs are in close contact with the skin and their surface area is clearly outlined. However, the patterned pigmentation of the dorsal skin surface of *Narcine* partially obscures this outline. On the ventral surface, not only is the total outline of the organ clearly visible, but also the honeycomb-like arrangement of the columns of electroplaques is seen through the skin. The main organ of each side extends laterally from the outer surface of the gill sacs to a clearly demarcated line near the edge of the pectoral fin. Rostrocaudally the organ starts slightly anterior to the eyes and extends back to the lines of the pectoral girdle.

Columns of electroplaques. As in other Torpedinidae the main organ is made up of a number of closely packed vertical columns, each column composed of electroplaques stacked one atop the other like a roll of coins. The packing of the columns leads to the honeycomb pattern mentioned above, but each column takes on an irregular rather than a hexagonal shape. In 10 adult specimens the number of columns ranged from 386 to 452 (average 419, Table I). This figure falls within the range previously given for this species (Bigelow and Schroeder, 1953; Cox and Breder, 1943; Fritsch, 1890). In late embryos and new-born specimens examined, the number of columns in the main organ was appreciably less than in the adults (Table I). This matter, important in the theoretical aspects of electric organ development, will be dealt with in more detail in the Discussion.

The array of electroplaques in a column. As in other Torpedinidae, the electroplaques in the main organ of *N. brasiliensis* are innervated only at their ventral surfaces. This was established both electrophysiologically (unpublished data from this laboratory) and anatomically. Electrophysiological data, employing successive penetration of the cells in a column with a microelectrode, indicated that the electroplaques were exceedingly thin. This was confirmed on histological preparations, the average thickness of the electroplaques being about 7 μ (*cf.* Fig. 3), although the surface they present is large (1.5 to 2 mm. in diameter, *cf.* Fig. 2). As with many other types of electroplaques, however, there is considerable extracellular material (Ballowitz, 1938; Ellis, 1913; Grundfest, 1957a; Luft, 1956; Szabo, 1956). Thus, there were only 486 to 541 cells (average 495) in the thickest (ca. 2 cm.)

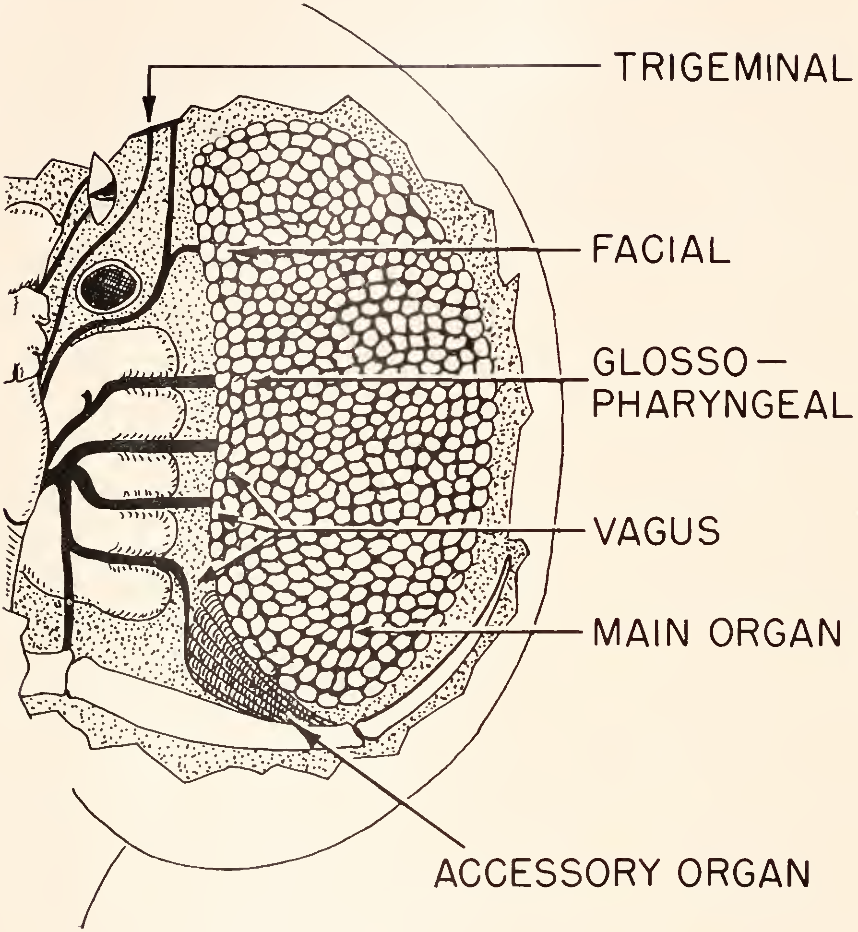
TRIGEMINAL
FACIAL
GLOSSO-PHARYNGEAL
VAGUS
MAIN ORGAN
ACCESSORY ORGAN

FIGURE 1. Electric organs and their innervation in *N. brasiliensis*. Dorsal view, partly schematic.

TABLE I

Number of electroplaque columns in main organ

Type of specimen	Number of specimens	Number of columns*	Average number
Adult	10	386, 381, 394, 400, 430, 440, 447, 450, 452	419
New-born	3	269, 287, 348	301
Embryo**	5	277, 297, 300, 348, 351	315

* In each fish the columns of one organ were counted three times. The figures given are averages.

** The embryos were all in a very late stage of development.

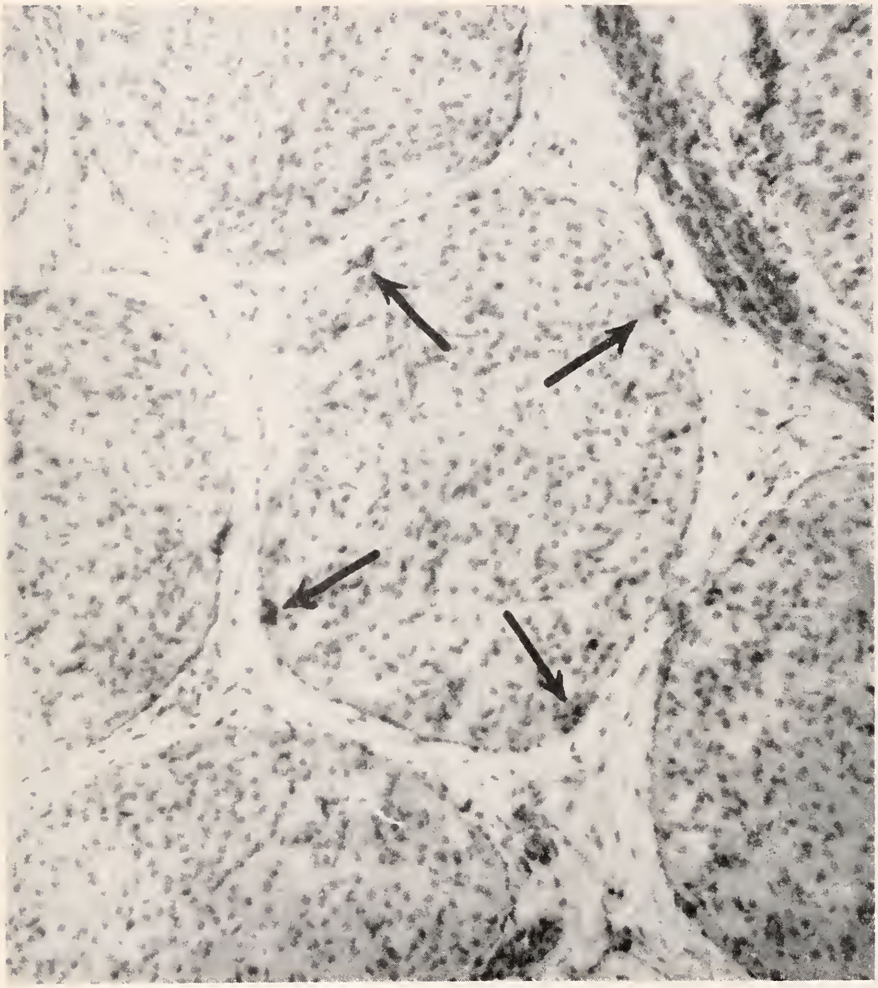

FIGURE 2. Innervation of individual electroplaque. Arrows point to four nerve fibers which become unmyelinated and disperse profusely over innervated surface. Fiber in lower left quadrant probably was cut parallel to its axis. Largest diameter of fiber is 2 μ.

portions of the main organ. In the shorter columns, nearest the outer edge of the fish, the average number of cells was 314.

B. Accessory Organs

Caudad to each main organ in *Narcine* there lies a smaller structure that both by electrophysiological and anatomical criteria has now been identified as another, hitherto undescribed, electric organ. This differs significantly in many respects from the main organ and therefore is termed an accessory electric organ.

Gross anatomy. The accessory organs arise dorsally in the articulation of the cartilaginous scapular process of each side. They run obliquely ventral and slightly

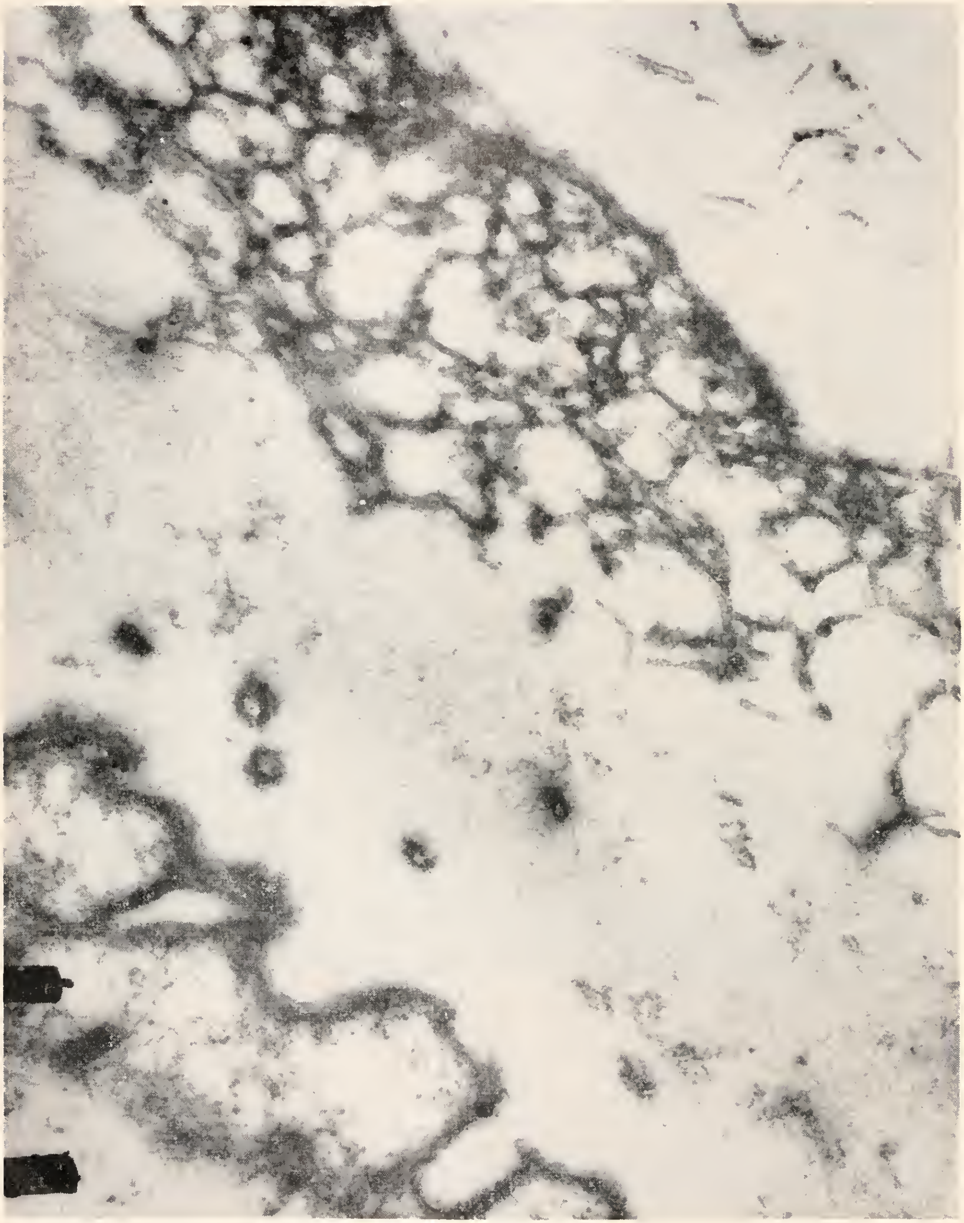

FIGURE 3. Electron micrograph of a slightly oblique cross section of electroplaque. Index (lower left) is 1 μ. Innervated surface, seen diagonally, at lower left, shows nerve fiber following involuted contour of cell membranes. Ring-like structures are sections of infolded fingers of innervated membrane. Interior of electroplaque shows fibrous material. Non-innervated face of cell (upper right) has complex system of interconnected canaliculi. Collagen fibers in extracellular space. Outlines of membranes not clear in this section which was cut thick (0.1 μ) to show other structures.

rostral, toward the midline, terminating in contact with the ventral skin immediately behind the main organs (Fig. 1). Each is about 1 cm. in diameter and about 2 cm. long in 30-cm.-long adult fish. It is separated from the main organ by a distinct layer of connective tissue.

Columns of cells. On the average, only 10 columns are found in the accessory organ. At the ventral surface the columns are tightly compressed and assume a honeycomb appearance which is also visible through the ventral skin. The cells in each are 2 mm. or slightly more in diameter. The area of each cell thus is not much larger than in the main organ and the anterior ventral margin of the accessory organ is not clearly differentiated from the adjacent main organ.

Cellular array in column. A striking feature, further differentiating the accessory organ, is the thickness of the individual cells, which is 20–30 μ, or 3 to 4 times greater than in the main organ. The number of cells in series in a single column is about 200, indicative of the large proportion of extracellular material also in this structure. Another difference between the main and accessory organs is the orientation of the innervation of the electroplaques. In the accessory organ, the dorsal surfaces of the electroplaques are innervated, the discharge of this organ producing positivity at the ventral surface of the fish (unpublished data from this laboratory) whereas discharge of the main organ results in negativity at the ventral surface (*cf.* Grundfest, 1957a). Responses to a single stimulation of the accessory organ produce only 0.5 to 1 volt, in comparison with the 25 to 35 volts generated during the discharge of the main organ.

C. Nerve Supply to the Electric Organs

Gross dissections of *Narcine*, as well as histological preparations, disclose some differences in the neural anatomy between this form and *Torpedo*.[5]

Gross anatomy. In *Narcine* the facial (VII), glossopharyngeal (IX), and vagus (X) nerves supply the electric organ (Fig. 1). The fifth cranial nerve (trigeminal) does not enter the electric organ, although in *Torpedo* it is reported to participate in the innervation (Fritsch, 1890; Szabo, 1955).

The facial, immediately after emerging from the skull, bifurcates into two large trunks. The cranial branch passes around the anterior periphery of the main electric organ and thence radiates throughout the head and pectoral fin. The remaining branch enters the electric organ immediately anterior to the first gill sac. The glossopharyngeal nerve, which emerges from the brain closely associated with the auditory nerve (VIII), leaves the skull ventral to the otic capsule. Passing between the first and second gill sacs, it immediately enters the electric organ. Of the total of four branches which comprise the vagus nerve, the first two pass between the second and third, and the third and fourth gill sacs, respectively, then entering the main electric organ. The third branch passes between the fourth and fifth gill sacs, runs along the anterior edge of the scapular process and enters the accessory electric organ. The fourth branch of the nerve runs parallel to the vertebral column back to a point near the pectoral girdle, then disappears into the musculature of this area.

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[5] But one specimen of *Torpedo ocellata* was available for comparison. However, published descriptions of *Torpedo* neuroanatomy are detailed (Bigelow and Schroeder, 1953; Fritsch, 1890; Szabo, 1955).

Innervation within the electric organ. Once having entered the electric organs, the nerves branch profusely in the connective tissue, as has been described for *Torpedo* by Fritsch (1890). Individual electroplaques are, in general, each supplied by four single myelinated fibers (Fig. 2). The fibers lose their myelin sheath close to the innervated surface, and their branching is extremely profuse (Mathewson and Wachtel, unpublished data). The synaptic contacts are dispersed over the innervated electroplaque surface, which is ventral in the main organ and dorsal in the accessory.

Detailed electron microscopic and histochemical studies on the *Narcine* electric organs will be reported elsewhere (Mathewson and Lehrer, unpublished data). Electron micrographs indicate a considerable degree of gross differentiation between the innervated and non-innervated faces of the electroplaques (Fig. 3). The nerves running along the innervated surface often make intimate contact with finger-like inpocketings of this membrane. Vesicular structures (de Robertis and Bennett, 1953; Robertson, 1957) are seen in the cytoplasm of the nerve terminals. At the non-innervated face the cell is profusely riddled by interconnected canaliculi, some of which appear to extend up to the tubules made by the inpocketing of the innervated surface.

DISCUSSION

Distinctions between the main and the accessory electric organs. Several differences in electrophysiological properties have already been indicated. A marked distinction is the reverse polarity of the activity in the accessory organ. The voltage produced by a single stimulation of the accessory organ is small, but grows rapidly upon repetitive stimulation (unpublished data from this laboratory). The large facilitation is in marked contrast to the behavior of the main organ and indicates important differences between the functional synaptic connections of the organs, which may be more clearly revealed after further histochemical and electron microscopic studies.

The anatomical distinctions are also marked, particularly the reversed surface of innervation and the greater thickness of the electroplaques of the accessory organ. The different orientation of this organ, with the columns running obliquely rather than dorsoventrally as in the main organ, and the delineation of the accessory organ by a distinct investment of connective tissue suggest that the muscles from which this organ is derived are different from those which are precursors of the main organ (Fritsch, 1890). Embryological material has not yet been studied sufficiently to reveal that origin.

Number of columns and electroplaques. Our counts of the number of columns in adult fish closely approximate other data in the literature. However, the embryos and new-born fish had a significantly smaller number of columns, although the number of cells in each of these columns was comparable with that in adults. This finding appears to contradict the delle Chiaie-Babuchin rule (du Bois-Reymond, 1881; Grundfest, 1957a) which states that the total number of electroplaques laid down is fixed early in development and does not increase thenceforth. It seems, rather, that the transformation of muscles (or of their anlagen) to electroplaques proceeds at unequal rates, more rapidly in the central portions of the main organ than at its periphery. It has been noted (Grundfest, 1957a) that regeneration of tissue takes place in knifefishes (Ellis, 1913) and this apparent contradiction of the dell Chiaie-Babuchin rule has been confirmed in this laboratory (unpublished).

In three adult *Narcine* Cox and Breder (1943) obtained an average count of 380 columns (range 343–416). The average in three embryos was 286 (range 264–340). These authors also concluded that the number of columns increased with growth of the fish. In two embryos, they also counted the number of cells in a column, finding an average of 305 in one and 482 in the other. The last, which is close to the value obtained in the present work from adults and embryos (500), supports the conclusion that the number of electroplaques in a column does not increase.

The higher values obtained in the present work help to remove a puzzling difficulty in electrophysiological data (Grundfest, 1957a). On the basis of the older figure for the series elements and of reported maximal discharge voltage, it had been estimated that the EMF of a single electroplaque of *Narcine* was about 120 mv. However, these cells do not produce spikes but only postsynaptic potentials (p.s.p.'s) (Grundfest, 1957a; and unpublished data from this laboratory). Unlike spikes which in skeletal muscle fibers and in eel electroplaques attain amplitudes of about 150 mv, the known varieties of depolarizing p.s.p.'s do not exceed the resting potential, which is generally some 60–80 mv. The maximal discharges in the fish used in the present experiments were about 35 volts in amplitude and this indicates that each electroplaque was capable of a maximal response of about 70 mv. This calculation approximates the resting potentials obtained in the cells, and accords also with direct observations on the responses of single electroplaques (unpublished data from this laboratory). Calculation for the e.m.f.'s of single electroplaques of *Torpedo marmorata* (Grundfest, 1957a) also gave very high values. It is likely that Fritsch (1890) also underestimated the number of cells arrayed in series in each column in these fish. *Torpedo* electroplaques are about 20 μ thick, but are densely packed (Luft, 1956, Fig. 4). It is very unlikely, therefore, that in these larger fish the series array (given as 400) is smaller than that found in *Narcine* (500).

Innervations of the electric organs. It has already been noted that the trigeminal nerve does not supply the electric organs in *Narcine*. According to Fritsch (1890) the *Torpedo* organs are innervated by the trigeminal and vagus nerves. However, he details an extensive controversy regarding this question. Other authors (*cf.* also Rosenberg, 1928) included the facial and hypoglossal in the nerve supply of the organs. Hunter's famous preparation (reproduced in Rosenberg, 1928) shows the electric organ of *T. occidentalis* supplied only by the nerves that would appear to be the glossopharyngeal and the first two branches of the vagus nerve.

Another difference between *Torpedo* and *Narcine* is the finding that the innervation of single electroplaques in *Narcine*, while as regular as is that pictured for *Torpedo* (Fritsch, 1890; Grundfest, 1957a), does not occur at six, but at four points of the cell surface. It seems likely that Fritsch (1890) overemphasized the hexagonal configuration in *Torpedo* and, indeed, in a number of illustrations of his monograph he shows surface views of the columns with shapes that are far from being regular or hexagonal.

Some functional correlations with the anatomical data. The dense synaptic innervation of the individual electroplaques in *Narcine* is in agreement with the similar data reported for *Torpedo* and *Raia* (Ballowitz, 1938; Grundfest, 1957a).

This would appear to have an important functional value. The cells generate only p.s.p.'s which are not electrically excitable and therefore do not propagate (Grundfest, 1957b). A maximal discharge of the organ would be produced only by simultaneous activation of a large proportion of the membrane. This result is achieved by dense synaptic terminations.

The usefulness of this densely innervated surface for a number of experimental purposes (Grundfest, 1957a) is augmented by the finding that the accessory organ has different kinetics of responsiveness to its neural stimuli. As noted above, this will provide a comparison material in the same preparations, not only for electrophysiological studies, but also for correlation of structure and function.

The preliminary electron microscopic data also suggest some possible correlations between function and structure. The current theories of muscle structure and of the contractile process (*cf.* Huxley, 1957) seek to account for transfer of activity from the electrically excitable membrane to the contractile elements by a special membrane extending into the muscle fiber and lying perhaps at the ~~electrically excitable membrane to the contractile elements by a special membrane extending into the muscle fiber and lying perhaps at the~~ Z lines. The dense canaliculi seen in the non-innervated aspect of the *Narcine* electroplaques might be the remnants of these structures, tubules which represent extensions of the membrane. The inpocketings of the opposite, synaptically excitable membrane may be continuous with the segments of the canalicular structures. A rather similar, but not so dense system is also found in eel electroplaques (Grundfest, 1957a; Luft, 1956) and in other types of electric organ (personal communication from Dr. Luft). Examination of electric organs in their various embryological states therefore may furnish important clues to the nature of the transfer from conductile to contractile activity.

Narcine electric organ also may be a favorable object to test current views (del Castillo and Katz, 1956; de Robertis and Bennett, 1953; Robertson, 1957) that the vesicles in the presynaptic terminals may represent concentrations of transmitter agent. Repetitive stimulation of the electric nerve of *Narcine* (Chagas *et al.*, 1953) rapidly blocks the response of the organ. With the period of unresponsiveness is correlated a large fall in the concentration of acetylcholine in the organ. The gradual return of responsiveness is also associated with a rise in the acetylcholine concentration. If the vesicles are the sites of transmitter storage, they should be subjected to marked changes upon repetitive stimulation and during subsequent recovery of responsiveness.[6]

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[6] For the use of the electron microscope and other facilities we are indebted to Drs. Leonard Ornstein and Allen Wachtel of the Cell Research Laboratory, Mt. Sinai Hospital, New York. Thanks are also due to Miss Ruby Tamura for preparing much of the tissue used in this study.

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THE OXIDATIVE METABOLISM OF EGGS OF URECHIS CAUPO[1]

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The cytochrome system is of such widespread occurrence in cells of aerobic organisms that reports of its absence in any particular case are of special interest.

There have been several instances in which spectroscopic detection of absorption bands of the cytochromes was at first reported to be negative and later shown to be positive when improved methods were employed. Thus in eggs of sea urchins Brachet (1934), Lindahl (1936), Krahl, Keltch and Clowes (1939) and Ball and Meyerhof (1940) reported that the cytochrome bands did not show up spectroscopically, although early evidence of inhibition of O_2-uptake by cyanide and by carbon monoxide (Runnström, 1930) indicated the operation of the cytochrome system, at least in the fertilized eggs. Later, Rothschild (1949), Borei (1951) and Yčas (1954), using the method of Keilin and Hartree (1939, 1949) of intensifying cytochrome bands by cooling the material in liquid air, were able to demonstrate the bands of cytochromes *a* and *b*.

It was also thought, at one time, that the respiratory system differed qualitatively in unfertilized and fertilized sea urchin eggs, the cytochrome system being inoperative in the former and brought into play upon fertilization (Korr, 1939). This was based on evidence of insensitivity of the respiration of unfertilized eggs to inhibition by cyanide and carbon monoxide (Runnström, 1930; Lindahl, 1939; Korr, 1937), and reported differences in the effect of temperature on the respiration of unfertilized and fertilized eggs (Rubenstein and Gerard, 1934; Korr, 1937). However, this evidence has now been largely contradicted. Thus, Robbie (1946b) showed that the O_2-uptake of unfertilized sea urchin eggs could be almost completely inhibited by low concentrations of cyanide when the precaution is taken of preventing the distillation of cyanide from the egg suspension to the center-well of the manometer vessel, by the use of appropriate $Ca(CN)_2$–$Ca(OH)_2$ mixtures in the center well. In regard to the effect of carbon monoxide on unfertilized sea urchin eggs, Rothschild (1949) was able to demonstrate a photo-reversible inhibition of O_2-uptake when account was taken of a CO-induced stimulation and a light-induced inhibition of respiration. Concerning the effect of temperature on respiratory rates, further measurements (Tyler and Humason, 1937; Borei and Lybing, 1949) have shown no significant differences between unfertilized and fertilized sea urchin eggs.

In eggs of the echiuroid worm *Urechis caupo*, a failure to detect the absorption bands of cytochrome was reported by Horowitz and Baumberger (1941). In these eggs there is a reversibly autoxidizable pigment which Horowitz (1940a)

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[1] This investigation was supported by a research grant (C-2302-C3) from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

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called urechrome. From the facts that both the oxidized and reduced states are observed naturally in the eggs, depending upon the presence or absence of oxygen, that the pigment is reducible by the cells, and that it autoxidizes in the physiological range of pH, he concluded that it was probably involved in the cellular respiration. Upon further characterization Horowitz and Baumberger (1941) suggested that the pigment was related to the hemins. Its chemical constitution has not as yet been determined.

For these and other reasons we decided to examine the O_2-uptake of fertilized eggs of *U. caupo* in the presence of cyanide and of carbon monoxide, and to examine the eggs spectroscopically using the low temperature method of Keilin and Hartree (1939). Previous measurements of the respiration of this material were made for other purposes (Tyler, 1936; Tyler and Humason, 1937; Tyler and Horowitz, 1938; Horowitz, 1940b) and it was noted that the fertilized eggs have rather consistent values for their absolute rate of O_2-uptake, although that of the unfertilized eggs may vary greatly from one batch to another. Some preliminary experiments with cyanide and azide were mentioned in the report of a seminar talk (Tyler, 1937). These were for the purpose of investigating possible correlations between cleavage retardation and respiratory inhibition and were done before the introduction by Robbie (1946a) of the $Ca(CN)_2$–$Ca(OH)_2$ center-well mixtures for preventing loss of cyanide from cell-suspensions in the manometer vessels. The preliminary experiments indicated, however, that inhibition of respiration by cyanide was obtainable in these eggs.

MATERIAL AND METHODS

General manometric procedure. Eggs of *U. caupo* were inseminated and washed in sea water buffered at pH 8 with 0.01 *M* glycylglycine, which Tyler and Horowitz (1937) showed to be a suitable non-injurious agent to replace the bicarbonate system of ordinary sea water. The latter system does not provide satisfactory buffering because the absorption of CO_2 by the alkali in the manometer vessels occasions a rise in pH of the sea water which is only partially and variably compensated by the CO_2 production of the respiring cells.

After examination to check that fertilization had been successful, 3- or 4-ml. aliquots of egg-suspension were transferred to standard Warburg-Barcroft manometer flasks whose calibration volumes were around 20 ml. Readings were taken after a 30-minute equilibration period in the water-bath, the shaker speed being 95 c.p.m. at 4 cm. stroke. The temperature was 20° C.

Cyanide experiments. Robbie's (1946a) $Ca(CN)_2$–$Ca(OH)_2$ mixtures, in 0.6-ml. quantity, were used in the center-wells of the manometer vessels in order to establish and maintain known concentrations of cyanide in the egg suspensions, and provide sufficient alkali to absorb the respiratory CO_2. Fluted filter papers were used in the center-wells to increase the absorbing surface. A stock 1.32 *M* calcium cyanide solution was prepared according to Robbie and Leinfelder (1945) and this was diluted with 10% $Ca(OH)_2$ according to Robbie's (1946a) figures to provide center-well mixtures establishing the following concentrations of HCN in the experimental fluid at 20° C.

HCN molarity	10^{-3}	10^{-4}	5×10^{-5}	10^{-5}	5×10^{-6}
Molarity of $Ca(CN)_2$ in 10% $Ca(OH)_2$	0.38	0.046	0.023	0.0054	0.0028

In some experiments an appropriate quantity of NaCN was added to the egg-suspensions in the manometer flasks just before the beginning of the experiment. In the latter case the equilibration-time was reduced from thirty to fifteen minutes. Manometer flasks and other vessels containing cyanide solutions were kept stoppered at all times except when eggs were added and the flasks were put on the manometers.

CO experiments. The gas phase of the manometers was filled with 95% CO in O_2 (95% CO/O_2), after flushing out the air. Ninety-five per cent N_2/O_2 and air controls were run at the same time. The center wells contained 0.3 ml. N/1 KOH and filter papers. Equilibration was in the dark for ten minutes.

RESULTS

Cyanide experiments. The results of three sets of experiments were clear-cut in the sense that, even at low concentrations, cyanide inhibited the respiration of fertilized eggs. Data from one of these are plotted in Figure 1. The lines labelled O,KOH and O,Ca$(OH)_2$ were controls to compare the CO_2-absorptive powers of 10% KOH and Ca$(OH)_2$ in the center-wells of the manometer flasks. As this and other tests showed, the Ca$(OH)_2$ proved as effective as the KOH in absorbing CO_2 under the conditions of these experiments.

TABLE I

The effect of cyanide, added 20 to 25 minutes after fertilization, on the percentage development of eggs of Urechis caupo, examined at 3 hours. The sea water contained 0.01 M glycyl glycine, pH 8.0, T° C. 20

Conc. HCN	Uncleaved	2-cell	4-cell	8-cell	16–32 cell	Unfertilized
10^{-4} *M*	99 } polar bodies					1
$5 \cdot 10^{-5}$ *M*	99					1
10^{-5} *M*	49	30	20			1
$5 \cdot 10^{-6}$ *M*	2	1	4	46	46	1
0	3				96	1

Table I shows the effects of the different concentrations of cyanide on the development of the eggs when examined at the end of the experiment.

Carbon monoxide experiments. The results of an experiment in which just-fertilized eggs were subjected to 95% CO/O_2 and 95% N_2/O_2 are shown in Figure 2, in which periods of illumination and darkness are indicated by black and white blocks along the time axis. If the rate of O_2-consumption[3] in the curve labelled CO/O_2 is examined by itself, it is clear that it rises upon illumination and falls in darkness in the manner considered characteristic of cytochrome-catalyzed respiration. When, however, comparison is made between the curve labelled CO/O_2 and the control labelled N_2/O_2, it is equally clear that, in the light, CO also stimulates the gas-uptake of these eggs. Illumination had no inhibitory effect on the O_2-uptake of eggs in equilibrium with air.

Table II shows the effect of CO in this experiment on egg development. The inhibition is not so marked as in the cyanide experiments, but it might, of course, be more dramatic if higher CO tensions were used.

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[3] The use of the terms O_2-consumption, O_2-uptake, and respiration in the description and discussion of the CO-experiments is subject to the qualification that there is the possibility (see Discussion) that some of the gas consumed might be CO.

The results of six sets of experiments with 95% CO/O_2 and 95% N_2/O_2 are presented in Table III. The last two columns of the table give a measure of the effect of CO on the respiratory rate, in the dark and in the light, based on lateral

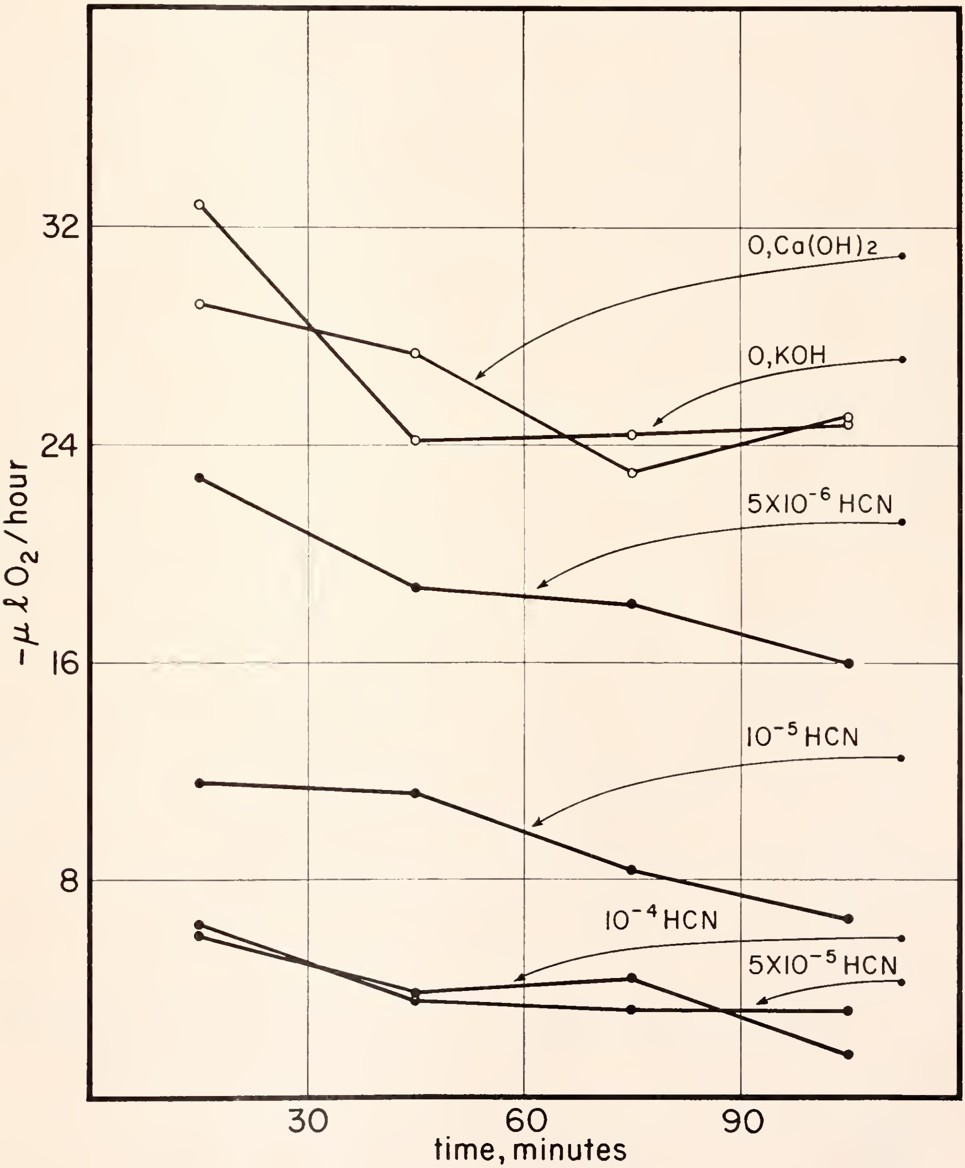

FIGURE 1. The respiration of eggs of *Urechis caupo* in the presence of HCN. For further details see text.

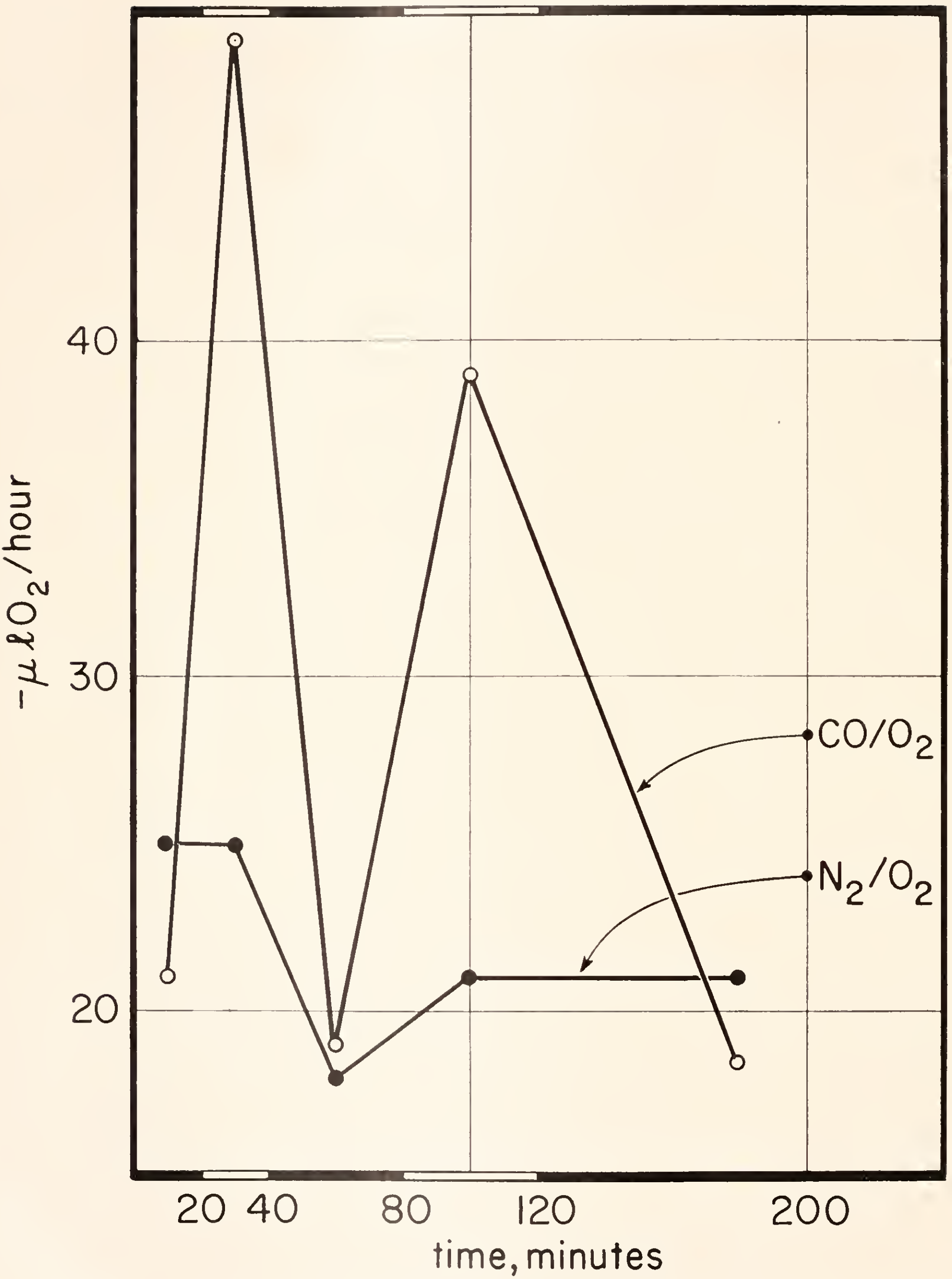

FIGURE 2. The oxygen uptake of eggs of *Urechis caupo* in the presence of 95% CO in O_2 and of 95% N_2 in O_2. The black and white blocks along the time axis correspond to periods of darkness and illumination. For further details see text.

TABLE II

The effect of 95% CO in O_2 and 95% N_2 in O_2 on the percentage development of eggs of Urechis caupo, exposed at ¼ hour and examined at 5 hours after fertilization. The sea water contained 0.01 M glycyl glycine, pH 8.0, T° C. 20

Gas	Uncleaved	64-cell	128-cell
Air	15	50	35
N_2	15	50	35
CO	15	85	

TABLE III

Effect of carbon monoxide on the respiration of eggs of Urechis caupo in the light and in the dark (All experiments started about 40 minutes after fertilization. Temp. 20° C.)

Experiment	Respiration period		Cu.mm. O_2 per hr. per 10^5 eggs		Ratios $\frac{\text{Resp. in 95\% CO–5\% } O_2}{\text{resp. in 95\% } N_2\text{–5\% } O_2}$	
			95% CO–5% O_2	95% N_2–5% O_2	Dark	Light
1	0′–15′	dark	7.6	9.7	0.78	
	15′–30′	light	14.6	9.7		1.59
	30′–45′	light	13.5	8.2		1.65
	45′–60′	dark	5.9	7.7	0.77	
	60′–75′	dark	4.9	6.7	0.73	
2	0′–20′	light	15.7	9.8		1.60
	20′–60′	dark	7.1	9.1	0.78	
	60′–100′	light	19.5	9.3		1.88
	100′–140′	dark	7.8	8.8	0.89	
	140′–160′	light	15.7	9.8		1.60
3	0′–21′	dark	7.5, 7.6	9.4, 8.7	0.83	
	20′–40′	light	17.1, 18.3	8.0, 10.1		1.95
	40′–80′	dark	6.8, 6.8	7.2, 6.1	1.02	
	80′–120′	light	13.5, 15.1	6.9, 8.4		1.87
	120′–140′	dark	6.9, 6.6	8.0, 7.5	0.87	
4	0′–30′	dark	8.7	11.8, 10.8	0.77	
	30′–60′	dark	9.6	11.1, 14.7	0.75	
	60′–90′	light	18.4	8.2, 9.3		2.10
	90′–120′	light	17.6	8.8, 8.5		2.02
	120′–240′	light	15.2	8.9, 8.9		1.71
	240′–270′	light	14.4	8.9, 8.5		1.66
	270′–300′	light	15.2	10.4, 10.0		1.49
5	0′–90′	light	15.8	6.4		2.47
	90′–150′	light	13.0	5.7		2.28
	150′–180′	light	14.1	5.7		2.47
	180′–240′	light	13.8	6.5		2.12
6	0′–60′	light	13.5	9.4		1.44
	60′–210′	light	15.4	10.9		1.41
	210′–240′	light	13.5	7.4		1.82
	240′–270′	light	11.8	5.7		2.07
	270′–300′	light	15.2	9.9		1.53

comparisons (*i.e.*, of different vessels run in parallel with aliquots of the same egg-suspension). In the dark the respiratory rate in 95% CO/O_2 is consistently lower than in 95% N_2/O_2. Rigid statistical treatment would be complicated because of the differences in times of readings, magnitude of respiration, etc., in the different experiments. However, a simple averaging of the percentage decrease (with double and quadruple weights for experiments 4 and 3, respectively) gives a 15 per cent inhibition of respiratory rate in 95% CO/O_2 in the dark.

Similarly calculated, there is in these experiments, in the light, an 85 per cent average increase in respiratory rate of the eggs in 95% CO/O_2 over that of the parallel controls in 95% N_2/O_2. The figures in Table III also show, for individual manometer vessels, the great effect of alternate light and dark periods on the respiration of the eggs in 95% CO/O_2 and the lack of significant effect of light and dark periods on the respiration of the eggs in 95% N_2/O_2.

Spectroscopic examination of eggs. We have examined the unfertilized eggs of *Urechis* with a narrow-dispersion hand spectroscope (Keilin, 1925) at the temperature of liquid nitrogen, the eggs being suspended in 50% glycerol (*v/v*) with sodium dithionite added (Keilin and Hartree, 1939, 1949, 1955). A double absorption band at 551 mμ, which is in the region of the α-band of cytochrome *c*, could be clearly seen. A further, faint, absorption band at 580–590 mμ (cytochrome *a*) was also seen. The presence of these absorption bands was confirmed by Professor D. Keilin and Dr. R. Hill.

Reduced cytochrome *c* was rapidly oxidized by egg brei in phosphate buffer. A peculiar phenomenon was observed during examination of the oxidation of cytochrome *c* by egg brei. When the oxidized cytochrome *c* and egg brei was kept in comparative darkness and then illuminated through the microscope sub-stage condenser (which automatically occurs during spectroscopic examination), the absorption bands of reduced cytochrome *c* gradually reappeared. This also was confirmed by Professor D. Keilin and Dr. R. Hill. The most probable interpretation is that in the presence of light, some reducing substance is produced by the eggs, causing the reduction of cytochrome *c*. This phenomenon may have some connection with the inhibitory action of light on the respiration of sea urchin eggs (Rothschild, 1949), though, as mentioned above, we have not observed any comparable light-inhibition of respiration in *Urechis* eggs. Certain dyes are affected by light in ways which would be consistent with the observed reduction of cytochrome *c* in light, which raises the possibility that urechrome may be concerned in the phenomenon. For example, Equ. (3) in Clare's article in Hollaender's *Radiation Biology*, Vol. III (1956)

$$DH_2 + O_2 \rightarrow H_2O_2 + D,$$

if written in the form

$$DH_2 + 2\ \text{cyt.c}^{3+} \rightarrow 2\ \text{cyt.c}^{2+} + D + 2H^+$$

is suggestive in this connection.

DISCUSSION

In the introduction to this paper, reference was made to Horowitz's (1940a) view that urechrome and not cytochrome catalyzed the respiration of *Urechis* eggs;

this opinion was based on the facts that urechrome is reversibly autoxidizable and that no absorption bands of cytochrome were observed. We have now shown that the absorption bands of cytochrome are present in these eggs and that an egg brei can oxidize reduced cytochrome *c*. Moreover, the inhibition studies with cyanide and carbon monoxide support the view that the respiration of these eggs is cytochrome-catalyzed. Just where urechrome fits into the picture is, at present, uncertain. The effects of CO and cyanide on this pigment have not, as yet, been studied.

The stimulating effect of carbon monoxide on respiration has been noted in many experiments with eggs and other tissues. The following citations from the literature on this subject will serve to illustrate the widespread occurrence of the phenomenon.

Runnström (1930) found that the respiration of unfertilized eggs of *Paracentrotus* and *Arbacia* was either not inhibited or somewhat higher in carbon monoxide-oxygen mixtures than in air, while that of the fertilized eggs was greatly inhibited. Presumably, although not explicitly stated, these experiments were run in the dark.

Lindahl (1939) obtained a 44% stimulation of the respiration of unfertilized eggs of *Paracentrotus* by 75% CO/O_2 in the dark, and this increased (to ca. 100%) upon illumination. With decrease in oxygen tension to 5% (+ 15% N_2 and 75% CO) the stimulation decreased. For freshly fertilized eggs in the dark he obtained a slight stimulation in 75% CO/O_2 and a marked inhibition in 95% CO/O_2. In the light the fertilized eggs showed marked stimulation by 75% CO/O_2 and this effect decreased as the O_2 concentration was dropped to 5% at constant CO.

Rothschild (1949) measured the respiration of unfertilized eggs of *Psammechinus miliaris* in various $CO–O_2$ mixtures. In 14 comparisons of the effect of 95% CO/O_2 with 95% N_2/O_2 in the dark there was no difference in two, an 11% decrease in three and a 14% increase in nine. Twenty-four comparisons of the effect of 95% CO/O_2 in dark with that in light showed a 44% increase in the light. At the same time he found an inhibitory effect of light on the respiration of the unfertilized eggs in air. This averaged 38% in 44 experiments. With 80% CO/O_2 in the dark there was an average of 55% increase in respiration above that in 80% N_2/O_2, and no significant change upon illumination.

In the ascidian *Phallusia mammillata* Minganti (1957) found an increase in respiration of the unfertilized eggs in 95% CO/O_2 in the dark and a further increase in the light. The fertilized eggs showed a 14% to 20% decrease in the dark, which is about the same degree of inhibition as in the present experiments, and an increase (up to 40%) in the light.

Bodine and Boell (1934) obtained CO-stimulation of respiration of diapause embryos of the grasshopper *Melanoplus differentialis* and no significant effect of light. A similar stimulation by CO was found by Wolsky (1941) in a bivoltine race of the silkworm *Bombyx mori*, but not (Wolsky, 1938) in pupae of *Drosophila melanogaster*. Wolsky (1938) attributes this difference to the pupal stage being one of great activity as compared with diapause. Schneiderman and Williams (1954) found that the respiration of diapausing pupae of the *Cecropia* silkworm was but slightly affected by high concentrations of carbon monoxide; further experiments (Harvey and Williams, 1958) demonstrated that a cytochrome system functioned in this material, the resistance to CO being accounted for by cytochrome oxidase being present in great excess relative to cytochrome *c*.

In non-embryonic tissue the most extensively studied examples of CO-stimulation of respiration were those first reported by Fenn and Cobb (1932a, 1932b) in skeletal and heart muscle of frog and rat. This stimulation occurs in the dark or diffuse daylight and, as shown by Schmitt and Scott (1934), is increased by strong illumination. Fenn and Cobb (1932b) adduced evidence to show that the CO was oxidized to CO_2 and this has been further substantiated by Clark, Stannard and Fenn (1950) by the use of isotopically labelled CO. The latter investigators (1949) also reported such oxidation of CO by the intact animal (turtles and mice).

In plants Daly (1954) obtained increases of about 20% to 30%, in 95% to 97% CO, with leaf tissue of the wild plum, *Prunus americana,* in the dark. From the results of experiments with labelled CO he concluded that the increased gas-uptake by the tissue represents a real stimulation of respiration rather than oxidation of CO to CO_2. He also found a rather high R.Q. (up to 1.33) for the extra gas consumed and therefore suggested that aerobic glycolysis was increased by CO to a greater degree than O_2-uptake. He cited cases of such stimulation of aerobic glycolysis by CO which have been reported in spinach (Ducet and Rosenberg, 1952[4]), carrot (Marsh and Goddard, 1939), and rat retina and mouse[4] chorion (Laser, 1937).

The above-mentioned investigations indicate that the stimulating action of CO on respiration is of wide incidence in cells and tissues of animals and plants. In some cases (skeletal and heart muscle of frog and rat) there is strong evidence that the extra gas-uptake is due to the oxidation of CO. In others (plum leaves) it appears to be due to the stimulation of endogenous respiration. In the case of the fertilized *Urechis* eggs, and the other cases that have been cited above, the mechanism of the stimulating action of CO is, as yet, unknown and would constitute an interesting area of further investigation. For the present purpose the demonstration of a light-sensitive action of CO on the gas-uptake of the *Urechis* eggs serves to support the other evidence presented that a cytochrome system is operative in this material.

One of us (R.) is indebted to the Biology Division, the California Institute of Technology, for their hospitality during the course of these experiments. We are indebted to Miss Mary Jones for technical assistance.

SUMMARY

1. The respiration and normal development of fertilized eggs of *Urechis caupo* are inhibited by low concentrtaions of HCN, 5×10^{-6} M. Known concentrations of HCN were established within the manometer flasks by the use of $Ca(CN)_2$–$Ca(OH)_2$ mixtures in the center-wells, with and without the appropriate amounts of NaCN in the egg suspensions.

2. The respiration of fertilized eggs was photo-reversibly inhibited by 95% CO in O_2. The inhibition of development was not so marked at this tension as in the cyanide experiments.

3. CO markedly stimulated the respiration of the eggs in the light. The occurrence of a similar action in the dark is presumed to account for the moderate degree of depression of respiration by CO in the dark.

[4] Daly (1954) cited a 1951 paper instead of the 1952 paper listed here; also he referred to chicken chorion whereas Laser (1937) refers to mouse chorion.

4. Spectroscopic examination of the eggs at the temperature of liquid nitrogen revealed absorption bands at 551 mμ and 580–590 mμ. Absorption bands at these wave-lengths are associated with the presence of cytochromes *c* and *a*.

5. An egg brei rapidly oxidized reduced cytochrome *c*, but intense illumination of the system reversed the process.

6. It is concluded that the respiration of *Urechis* eggs is cytochrome-catalyzed.

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REGENERATION OF BUDS IN BOTRYLLUS[1]

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The process of budding in the colonial ascidian, *Botryllus schlosseri*, has been carefully analyzed by N. J. Berrill (1941a) and recently by Sabbadin (1955). The new buds (Z_3) arise from the atrial epithelium and epidermis of large buds (Z_2) in which internal structure is nearly complete but which are still attached to the parent (Z_1). The disc-like thickening of the atrial epithelium increases in cell number and area until a certain size, called the maximum disc, is reached. It then folds out into a hemisphere and finally to a closed sphere attached to the large bud by a stalk. Three generations are thus present and connected together at one time. The sphere then goes through a process of expansion, folding, and evagination to form the internal structure of the new zooid. The bud continues to grow until it reaches a size nearly equal to the parent, at which time the latter degenerates and the bud becomes functional. There is considerable variation among colonies in the number of buds formed and the number which reach maturity.

Berrill (1941a, 1941b, 1945) has shown that in young colonies the diameters of the maximum disc and sphere stages are less than half those of older colonies and that they gradually increase with each successive generation. The size of the adult zooid is closely related to the size of the bud and hence to the number of cells initially present. A sphere with a diameter of 0.035 mm., for example, becomes a zooid with a length of 1.1 mm., while a sphere of 0.080 mm. becomes a zooid of 2.6 mm. Sabbadin (1956a, 1956b) showed that the growth of the bud is conditioned not only by its initial dimensions, but also by the quantity of food made available to it by the regression of the parent zooid and the duration of its growth period. In his experiments all but one bud was removed from each zooid in the experimental colonies. These buds and the zooids from them attained greater maximum length than corresponding buds and zooids in control colonies. Sabbadin concludes that the buds on one zooid compete for food made available by the parent as it regresses. This does not explain, however, the gradual increase in size of the zooids with each generation. It was deemed of interest to determine whether the size of the zooid depends directly on the number of cells present in the early bud or is determined in some other manner by the parent. In order to study this problem, buds were damaged at an early stage and the amount of regeneration as shown by the final size was noted.

This degeneration of the parent zooid has generally been thought to be due to the increasing need of the bud for space and nourishment (Berrill, 1935). To test

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[1] This work was done while the author held an Anderson Summer Fellowship from the University of Minnesota Graduate School as a part of the Embryology Course given at the Marine Biological Laboratory, Woods Hole, Massachusetts. The author wishes to thank Dr. Mac V. Edds, Jr. for his help with both the research and the manuscript. Dr. N. J. Berrill read the manuscript and offered valuable advice.

whether this is true the buds were removed in an attempt to prolong the life of the zoöid.

Materials and Methods

Adult colonies of Botryllus were collected from the dock in Eel Pond at Woods Hole, Massachusetts, and placed in finger bowls on a table of running sea water. Each day a few of the tadpole larvae were released from the colonies and these were collected and placed in Syracuse dishes for approximately 12 hours. After the tadpoles had attached to the glass, the Syracuse dishes were inverted in wooden racks placed in tanks of running sea water. The tadpoles metamorphosed in less than one day, forming oozoids with large right buds which became the first blastozooids. Under these conditions at 22.5 ± 0.5° C. the adult zooid persisted for from 4 to 6 days, with as much as 24 hours' variation between two colonies in the same dish. The experimental colonies were examined every day or two with a binocular dissecting microscope and rough sketches were made to follow the fate of individual zooids and buds.

Buds were removed by cutting through the stalk with a needle sharpened to a blade. With care this could be done with very little damage to the parent, but sometimes the latter was damaged severely and disappeared. To determine whether the new buds originated from the same area as the destroyed buds or from bud primordia posterior to it, all the small buds (Z_3) were cut off 220 large buds (Z_2) in 31 colonies. The site of formation of the new buds was then observed. In 5 colonies an attempt was made to keep the parent zooids from degenerating by constantly removing new buds as they appeared.

Buds were damaged with sharpened steel needles inserted through the tunic. An effort was made to destroy half or more of the forming bud. Although the amount of actual damage varied from bud to bud, in most cases at least half of the bud was destroyed. Frequently, part of the bud was torn away and could be seen sticking to the needle. In preliminary experiments on 21 colonies, buds ranging from the sphere stage to those with some internal structure present were damaged. These were watched to see whether they reached maturity, but no measurements were made to determine if they were full size. In order to examine the effect of destroying approximately half of the cells at a stage before the closed sphere, both right and left buds were damaged when the atrial epithelium had begun to fold out into a hemisphere (between stages 2 and 3 of Berrill, stage 2 Sabbadin). The length of the zooid which formed these buds was then measured with an ocular micrometer and compared with the length of undamaged zooids. The width of the zooids varied in different colonies of the same age and seemed to decrease as the number of zooids around the cloaca increased; therefore, no measurements of width were made.

Results

a) *Degeneration of parent zooids*

In no case observed did an adult zooid persist beyond 24 hours of the time of degeneration of other zooids of the same age. When all the buds were removed from a zooid, that zooid degenerated at the same time as the rest of the zooids in that colony or in other colonies, whether new buds formed or not. The five colonies in which new buds were constantly removed as they appeared degenerated and disappeared within 24 hours of the time of degeneration of control colonies.

b) *Formation of new buds*

If the large bud which normally occurred on the right side of an oozoid was removed, a bud then appeared in four cases out of five on the left side of the oozoid and became a normal blastozooid. In later generations, if all the large buds (Z_2) with complete internal structure were cut off (4 colonies), the colony degenerated. If, however, only approximately half of the larger buds were cut off (5 colonies), those remaining proceeded to maturity, and in addition a few new buds appeared. When all the small buds (Z_3) at the sphere stage were cut off 220 large buds (Z_2) in 31 colonies, a total of 72 new buds appeared, an average of one bud for every three parents. There was a great deal of variation among the colonies, with anywhere from zero to seven buds produced by the six or seven parents. Of the new buds, 26 appeared on the left side of the blastozooids and 29 on the right side. Of the latter 6 definitely were from the same area as the destroyed bud, 18 were probably from this area, and 5 appeared posterior to the destroyed bud. The origin of the other 17 buds was impossible to determine. Most of these were first seen in the midst of a degenerating colony quite separate from any blastozooid.

c) *Bud regeneration after damage*

In preliminary experiments in which 107 buds ranging from the sphere stage to those with some internal structure present were damaged, 55 reached maturity. The rest of the buds became progressively smaller and eventually disappeared. In 36 colonies in which 361 hemispheres were damaged, 40% reached maturity as compared to 78% in 8 control colonies with 124 hemisphere stages.

TABLE I

Size regulation of zooids in partially damaged colonies

Colony number	No. of zooids in colony	No. of zooids damaged	Average length of *all* zooids in colony, in mm. ±S.D.	Average length of *damaged* zooids in colony, in mm. ±S.D.	Average length of *undamaged* zooids in mm. ±S.D.
A12c	12	5	1.5 ± .1	1.6 ± .1	1.5 ± .1
A12e	7	3	1.8 ± .1	1.8 ± .2	1.9 ± .1
A12h	21	8	1.9 ± .1	2.0 ± .1	1.8 ± .1
A12k	13	6	1.6 ± .1	1.6 ± .1	1.6 ± .1
A12l	17	4	1.4 ± .1	1.5 ± .1	1.4 ± .1
B2d	25	4	1.8 ± .2	1.8 ± .1	1.8 ± .2
A5a	8	3	1.8 ± .1	1.8 ± .2	1.9 ± .1
A5e	15	13	1.6 ± .1	1.6 ± .1	1.6 ± .1
A5i	7	6	1.8 ± .1	1.8 ± .1	1.9
A1a	16	16		1.8 ± .2	
A5b	6	0			1.4 ± .1
A5g	13	0			1.6 ± .1
A5h	13	0			1.7 ± .1
A6a	7	0			1.8 ± .1
A6b	11	0			1.7 ± .1

TABLE II

Size regulation of zooids in experimental and control colonies

Colony number	No. of Z_1	Length of Z_1 in mm. ±S.D. at time of experiment	No. of Z_2	Length of Z_2 in mm. ±S.D. 1–2 days after reaching maturity	No. of Z_3	Length of Z_3 in mm. ±S.D. 1–2 days after reaching maturity	Length of Z_3 in mm. ±S.D. 3–4 days after reaching maturity
Experimental Colonies							
A3e	9	1.7 ± .1	8	2.0 ± .2	12	2.0 ± .2	2.4 ± .3
A7a	5	1.8 ± .1	8	2.0 ± .1	12	2.2 ± .1	2.8 ± .1
A8a	7	1.7 ± .1	4	1.7 ± .1	4	1.9 ± .1	2.4 ± .1
A10a	4	1.6 ± .1	4	—	8	2.2 ± .1	—
A10e	—	—	6	1.7 ± .1	10	1.8 ± .1	—
Total	25	1.7 ± .1	30	1.9 ± .2	46	2.1 ± .2	2.6 ± .3
Control Colonies							
A3d	6	1.8 ± .1	8	1.8 ± .2	13	1.8 ± .1	—
A7b	5	1.6 ± .2	9	1.8 ± .1	15	1.9 ± .1	2.3 ± .2
A8a	7	1.8 ± .1	10	2.0 ± .1	17	2.0 ± .1	2.6 ± .1
A10b	5	1.8 ± .1	5	1.8 ± .1	11	2.1 ± .1	—
A10f	—	—	5	1.7 ± .1	9	1.8 ± .2	—
Total	23	1.7 ± .1	37	1.8 ± .2	65	1.9 ± .2	2.4 ± .2

d) *Size regulation in damaged buds*

In the first experiments, only some of the hemispherical buds in each colony were damaged with the idea of using the others as controls. The data for 15 such colonies are given in Table I. In colonies A12h and A121 the damaged left buds did not survive, so the right buds measured received the full food supply from the parents. In all other colonies as many damaged left buds survived as undamaged, so the supply of food did not affect the results. The colonies are not all of the same size or age at the time of the experiments, so the average length for different colonies cannot be directly compared, but the average lengths of damaged and undamaged zooids in the same colony show no significant difference.

In later experiments, all the hemisphere stages in a colony were damaged and these colonies were compared with control colonies. Three generations were present at the time of the experiment: the parent zooids (Z_1), the large buds (Z_2), and the hemispherical buds (Z_3). Each of these was measured as it in turn reached maturity. In both experimental and control colonies, most of the left buds reached maturity, so the food supply was about the same for all buds. The data for these experiments are given in Table II. No significant difference can be seen between the experimental and control colonies.

DISCUSSION

If the degeneration of the adult zooid is due only to the increasing need of the growing buds for space and nourishment, removal of all the buds in a colony ought to have prolonged the life of the zooids. In this study, any attempt to postpone degeneration of the zooid in this way met with failure. No zooid was observed to persist more than 24 hours longer than other zooids of the same age even if its buds were continually removed. Sabbadin (1956b), however, found that when all but one bud was removed from each zooid, that zooid had a prolonged stage of functional maturity. Perhaps removal of all buds was a shock to the zooid and partially caused its regression; however, it seems likely that adult regression will occur without the presence of buds. At the same time the buds may play an important part in the process by their increasing need for nourishment.

There is some question as to the origin of new buds after removal of these already growing. Blastozooids have two potential budding areas, one on the right side and one on the left, although frequently only the bud on the right side reaches maturity. At times, a third bud may be formed posterior to the bud normally found on the right side (see Watterson, 1945, and Sabbadin, 1956a, for a discussion of the number of buds usually formed). It would appear that a certain amount of atrial epithelium is set aside for bud formation; after that is used no more buds can be formed. Frequently in these experiments, when buds were removed from the blastozooid, new buds were formed at the same area as the buds were destroyed. Sabbadin (1956a) reports that he never observed buds arising "de novo" after removal of buds present. Sometimes, however, after he had removed buds in the hemisphere stage, he saw fragments adhering to the atrial side of the parent zooid, and these fragments formed new buds. This is a possible explanation of the present results although every effort was made to remove the entire bud intact. In these experiments all the buds removed were at least in the closed sphere stage and many were quite large and visibly separated from the parent though still attached by the stalk. It would take considerable powers of regeneration for fragments of such buds to form a whole new zooid.

The 17 buds whose origin it was impossible to determine might possibly be cases of vascular budding (Oka and Watanabe, 1957). They arose during or after the regression of the adult zooids, so a vascular origin seems likely. They were not observed, however, until they were large enough to obscure their point of origin.

Although development of ascidians from egg to tadpole is determinate (Conklin, 1905), the adults have remarkable powers of regeneration (Berrill, 1951). Zhinken (1939) has shown that while tadpoles have little ability to replace lost parts, the oozooid has acquired considerable regulative powers. The present study would indicate that buds also have the ability to regenerate lost tissues from the earliest stages onward.

Berrill has shown (1941b, 1941c, 1945) that the size of the maximum disc and sphere stages increases with succeeding generations and that the size of the adult zooid is clearly related to the number of cells or the diameter of the maximum disc and sphere. This might suggest that the parent determines the size of the new zooid by the number of cells which are initially incorporated into the early stages of the bud. If this were true, then destroying some of these cells would have resulted in smaller adult zooids. However, using length as an index of zooid size, it was found that there was no decrease in size of the zooids damaged at the hemisphere stage. After a bud was damaged it either disappeared completely or reached the predetermined size. Thus the size of the adult does not appear to depend directly on the number of cells originally present since these cells can be replaced. Either the parent zooid retains control over the growth of the bud or the bud has "received instructions" as to the size it should attain and follows them by regenerating lost tissue and then continuing to grow. Sabbadin (1956c) has shown that zooids with the position of the digestive tube reversed may appear if the growth of the bud is delayed at an early stage. The buds on these abnormal zooids showed a marked tendency to be the same as their parents unless the parent has started to regress before organogenesis is complete. This would indicate that the parents do retain control over the growth and organogenesis of their buds.

SUMMARY

1. The degeneration of the adult zooid of *Botryllus schlosseri*, which normally occurs when the buds become functional, occurred even after all buds were removed.

2. All stages of the growing buds of Botryllus have considerable regenerative ability.

3. Buds damaged in the hemisphere stage became adult zooids with the same length as undamaged zooids of the same age. Control over the size of the adult zooid appears to be maintained during the growth of the bud.

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Vol. 115, No. 2 October, 1958

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE OXIDATION OF CARBON MONOXIDE BY FERTILIZED EGGS OF URECHIS CAUPO SHOWN BY USE OF A C^{13} LABEL[1]

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In some previous experiments (Rothschild and Tyler, 1958) with eggs of *Urechis*, it was found that the rate of respiration in the presence of carbon monoxide (95% CO : 5% O_2) in the light was greatly increased above that of the controls (95% N_2 : 5% O_2). The average increase amounted to 85 per cent. In the dark there was a slight decrease, averaging about 15 per cent.

In many earlier investigations on eggs and other tissues of various animals and plants there have been reports of a stimulating action of CO on respiratory rate. Examples of this are found in experiments on eggs of sea urchins by Runnström (1930), Lindahl (1939) and Rothschild (1949); on ascidian eggs by Minganti (1957); on diapausing grasshopper- and silkworm-embryos by Bodine and Boell (1934) and by Wolsky (1941); on skeletal and heart muscle of frog and rat by Fenn and Cobb (1932a, 1932b), Schmitt and Scott (1934), and Clark, Stannard and Fenn (1950); on leaf tissue of the wild plum by Daly (1954).

In the experiments on vertebrate muscle tissues, Fenn and Cobb (1932b) and Clark, Stannard and Fenn (1950) obtained evidence that CO is oxidized to CO_2. Clark *et al.* (1949) also reported that intact whole turtles and mice could effect such oxidation of CO when this was administered at very low tensions. In the experiments on plum-leaves, on the other hand, Daly (1954) found that the increased gas-uptake in the presence of CO represents a stimulation of ordinary respiration rather than an oxidation of the CO. The question of whether or not the stimulation of respiration in eggs of sea urchins and ascidians is due to oxidation of the CO was considered by Lindahl (1939), Minganti (1957) and Rothschild (1949). The former two investigators rejected this view while the latter considered it to be the most probable explanation of the increased respiration. In a review of various experiments Runnström (1956) concludes that the evidence is against the possibility of oxidation of CO by sea urchin eggs. However, there has as yet been no direct test of this proposition, such as would be provided by the use of isotopically labelled CO.

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[1] This investigation was supported by a research grant (C-2302) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

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In the present experiments C^{13}-labelled CO was employed in an investigation of the possibility of its oxidation by eggs of *Urechis*. The results show that such oxidation occurs and that it accounts for all of the extra gas-uptake of the eggs in the light. The data also show that an oxidation of CO occurs in darkness, but at a lower rate.

MATERIAL AND METHODS

Eggs of the gephyrean worm *Urechis caupo* were employed in these experiments. They were inseminated in sea water and washed in sea water buffered at pH 8 with 0.01 *M* glycylglycine (Tyler and Horowitz, 1937).

Gas-uptake was measured with Warburg-Barcroft manometers using vessels whose calibration volumes ranged around 25 ml. The vessels generally contained 3 ml. of egg suspension and 0.3 ml. of *M*/1 KOH (low in CO_2). In some experiments in which CO_2 was to be released from the egg suspension as well as from the alkali, magnetically held cups were employed, one for the alkali and one containing 0.3 ml. of 6 *M* H_2SO_4. The contents of these could be separately tipped into the egg suspension at the desired time by removal of an externally supported magnet. The KOH used in the alkali-wells of the manometer vessels was prepared from a saturated solution, in which K_2CO_3 is largely insoluble, and diluted with CO_2-free double-distilled water under CO_2-free air. An analysis of the alkali prepared in this manner gave 0.8×10^{-6} mole of total carbonate per 0.3 ml. In filling the manometer vessels the alkali was introduced last.

The use of C^{13} offers some advantages over C^{14} for these experiments. Use of C^{14} would involve precipitating and weighing very small quantities (less than 4 mg. as $BaCO_3$) of the carbonate derived from the respired CO_2 in the alkali well of the usual manometer vessels. This is unnecessary for the mass spectrometric measurement of C^{13} which provides the required quantitative data in the form of the ratio of C^{13} to C^{12} in the sample. It also avoids such uncertainties as are entailed by the self-absorption of radiation in the measurement of C^{14}. In addition, use of C^{13} eliminates possible health-hazards and possible effects of radiation on the system under investigation.

The labelled carbon monoxide was prepared from barium carbonate containing 3.85% C^{13}. This was obtained from the Stable Isotopes Division of the Oak Ridge National Laboratories. The method employed was essentially similar to the continuous flow technique described by Bernstein and Taylor (1947). The apparatus consisted of a CO_2 generator connected to a Pyrex combustion tube (8 mm. i.d.), containing about 50 grams of zinc-dust-asbestos fiber (95 : 5), within a combustion-furnace of 18 cm. length, and leading through a three-way stopcock to the top of a storage bulb. The latter was provided also with a bottom stopcock leading to a levelling bottle containing N/10 NaOH. At the start of the preparation the storage bulb was filled with the alkali up to the three-way stopcock. A weighed amount of the C^{13}-enriched $BaCO_3$ was placed in the generator, and the generator and combustion tube, up to the three-way stopcock, were flushed with unlabelled CO. The furnace was set at 520° C. Hydrochloric acid was introduced into the generator at a rate producing about 25 to 50 cc. of CO_2 per minute. Measurements of the volume of fluid displaced in the storage bulb showed that the amount of CO obtained in this system was close to that expected. After CO_2 generation had stopped, the gas remaining in the generator and combustion tube was flushed into the storage bulb with enough unlabelled CO to make a final volume of one liter. The relative volumes of labelled and unlabelled CO were 362 to 638 for the preparation, giving a C^{13} content of 2.14%. Relative to a C^{13} content of 1.17% found for the CO_2 from *Urechis* eggs respiring in air, this gives 82.9% for the atom percentage excess C^{13} of the preparation. The labelled CO was stored over alkali for at least one day prior to use. Storage over alkali for several weeks showed no change in gas volume, indicating no significant contamination by acidic gases.

After attachment of the Warburg vessels to their manometers they were flushed with one liter or more of oxygen. They were then attached to a Toepler pump and evacuated to one-fifth of the original pressure, precautions being taken, by stoppering the open end of the manometers and closing-off the bottom rubber well with a clamp, to avoid drawing the Brodie's fluid out of the manometers. The C^{13}-labelled CO was then introduced through the three-way stopcock at the top of the manometers, after a preliminary flushing of connecting tubes. By this procedure the CO–O_2 ratio could be fixed with considerable accuracy to the desired value, which was 4 : 1 in the present experiments. About 15 minutes were required for these procedures and 10 minutes were allowed for equilibration in the temperature bath. The control vessels were left open to air during the gassing of the experimental vessels. The experiments were run at 20° C. Shaker speed was 95 c.p.m. at 3-cm. stroke. Illumination was provided by a bank of 30-watt reflector-type G-E incandescent lamps located below a glass shelf of the water bath. This supplied 1100 to 1200 foot-candles at the level of the egg suspensions in the Warburg vessels.

The C^{13} determinations were made with a Nier mass spectrometer (Nier, 1947) modified for detection of relatively small enrichments by McKinney *at al.* (1950). The sensitivity of the instrument is such that differences of two parts in ten thousand in the C^{13}-to-C^{12} ratios can be readily detected. For introduction of the respired CO_2 into the mass spectrometer, the procedure followed in two of the experiments (No. 1 and No. 2) was to transfer the alkali from the center well of the Warburg vessel quantitatively, with CO_2-free water and with precautions to avoid contamination with atmospheric CO_2, to a reaction vessel wherein the CO_2 could be liberated by tipping-in concentrated H_3PO_4 from a side-arm (McCrea, 1950). This was attached to the vacuum-line of the mass spectrometer. In one of the experiments (No. 3), the CO_2 was liberated within the Warburg vessels by tipping acid from one of the contained insert-wells into the egg suspension and the alkali. After measurement of their amounts the CO_2 samples were transferred to the reaction vessels by means of the Toepler pump. In two of the experiments (No. 2 and No. 3) a measured amount of $NaHCO_3$ was added to the reaction vessel in order to decrease the C^{13} enrichment to values within the range best suited for the mass spectrometer. The measurements are corrected for the dilution factor.

RESULTS

Effect of CO on gas-uptake of eggs of Urechis

The relevant respiration-data for three experiments are presented in Table I. The first two are for eggs run in the light starting shortly after fertilization, and the third is a dark-experiment with eggs at a similar period of development.

The increase in gas-uptake reported by Rothschild and Tyler (1958) for freshly fertilized eggs of *Urechis* in the light in 95% CO/O_2 is shown also in the present experiments (No. 1 and No. 2) with 80% CO/O_2. Likewise, the lack of appreciable inhibition in the dark is shown in the results of experiment No. 3. Examination of the eggs at the end of the respiration runs in experiments No. 1 and No. 2 showed no significant difference in rate of development between those in 80% CO/O_2 and those in air. The eggs from experiment No. 3 were not available for examination because of the acidification, but separate experiments on eggs run in the dark in $CO–O_2$ mixtures show only a small amount of inhibition of development, as reported previously (Rothschild and Tyler, 1958).

The data in Table I present amounts of gas-uptake calculated as if the total gas were oxygen. Part of the gas-uptake of the eggs in the $CO–O_2$ mixture could (and, as later shown, does) represent disappearance of CO. However, calculations using the solubility of CO instead of O_2 in the usual formula for converting the manometric pressure difference into volume of gas would change these figures by only 0.1%, since the solubility coefficients of the two gases are of the same order of magnitude and this factor contributes relatively little to the vessel constant. This difference is negligible here.

Experiments No. 1 and No. 2 give values of 154 and 130 mm³., respectively, for the excess gas uptake. Assuming that this is due to the oxidation of CO ($2\,CO + O_2 \rightarrow 2\,CO_2$), then ⅔ of these quantities represent the amounts of CO oxidized and the corresponding amounts of CO_2 produced therefrom; namely, 102 and 87 mm³., respectively. The corresponding control vessels yield 318 and 305 mm³. of CO_2, respectively, on the basis of an R.Q. of unity (Horowitz, 1940). The percentage of the CO_2 derived from oxidation of CO would therefore be 24.3 for experiment No. 1 and 23.4 for experiment No. 2. These are entered in the last column of Table II as expected values, and involve also the assumption that in the light there is no inhibition of the ordinary respiration.

TABLE I

Respiration-data for eggs of Urechis used in C^{13}-labelled CO experiments

(1) Experiment	(2) Number of eggs per flask	(3) Time interval of experiment in hours after fertilization	(4) Total gas-uptake In air (mm.³)	(5) Total gas-uptake In 80% CO/O_2 (mm.³)	(6) Excess gas-uptake in 80% CO/O_2 (mm.³)
1 (light)	389,000	$1\frac{1}{2}$–$8\frac{1}{2}$	318	472	+154
2 (light)	622,000	$1\frac{1}{2}$–$6\frac{1}{2}$	305	435	+130
3 (dark)	421,000	1–10	408	394	−14

TABLE II

Percentage of respired CO_2 derived from oxidation of CO, as determined from measurements of C^{13} in mass spectrometer and as calculated on the assumption that such oxidation accounts for all excess gas-uptake in CO-O_2 mixtures in the light

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Experiment No.	Atom % excess C^{13} in CO used in gas space of manometer vessels	Mass spectrometer data				Expected percentage of total CO_2 derived from CO as calculated from excess gas-uptake in light
		Atom % excess C^{13} in respired CO_2			Percentage of CO_2 derived from oxidation of CO	
		With reference to standard CO_2		With reference to control		
		Experimental vessel	Control vessel	Experimental vessel		
1 (light)	82.9	19.92	0.61	19.31	23.3	24.3
2 (light)	82.9	20.28	0	20.38	24.5	23.4
3 (dark)	82.9	16.23	1.35	14.88	18.0	

A calculation of expected CO-oxidation cannot be made in this way for experiment No. 3 which was run in the dark, wherein both inhibition of ordinary respiration and oxidation of the CO might well take place.

Mass spectrometer data relating to oxidation of CO

The results of determinations of C^{13} abundance in the respired CO_2 of the above three experiments are presented in Table II. The atom percentage excess C^{13} in the CO used in these experiments is listed in the second column of the table. These figures also represent the excess that would be expected if all of the respired CO_2 were derived from oxidation of CO. The values obtained from the mass spectrometer measurements for the excess C^{13} in the CO_2 from experimental, relative to that from control vessels, are given in the fifth column of the table. Division of these figures by the corresponding ones of column two gives the percentages (column 6) of the CO_2 derived from CO-oxidation in these three experiments. Comparison with the expected percentages (column 7) calculated from the manometrically determined extra gas-uptake, on the assumption that all of this surplus in the light is derived from CO-oxidation, shows close agreement in experiments No. 1 and No. 2.

This closeness of agreement may, however, be largely fortuitous as the following considerations of further details of the experiments indicate. In experiment No. 1 the control was an aliquot of the same egg suspension respiring in air. The alkali from both experimental and control flasks was transferred quantitatively to the reaction vessels and no carrier $NaHCO_3$ added. The respective percentages of excess C^{13}, relative to the standard used in the instrument, are given in columns 3 and 4 of the table. The air-control shows a small excess of C^{13} relative to the standard source. This simply reflects variation in C^{13}/C^{12} ratios of living and non-living materials from various sources (*cf.* Craig, 1953). Since the carbon of the respired CO_2 of the air-control is all derived from the eggs this indicates a higher C^{13} content in the eggs than in the standard. In the absence of other information the best method of applying a correction for the control is uncertain, but it seemed most reasonable to us simply to subtract it from the value for the experimental flask. In any case this correction has relatively little effect on the calculations of CO-oxidation.

In experiment No. 2 the respired CO_2 from the air-control vessel was not subjected to C^{13} analysis. Instead, a second type of control was investigated. This consisted of a preparation of lyophilized eggs that was run along with the experimental flask in the 80% labelled CO-20% O_2 atmosphere in the light. This preparation showed a negligible amount of gas-uptake, and was employed to test for possible exchange of carbon atoms between CO_2 and the labelled CO. For this purpose about 300 mm³. of CO_2 were introduced into the Warburg flask. The analysis of the CO_2 in the alkali of this flask showed no difference in C^{13} content from that of the standard. This indicates that no significant exchange of carbon atoms between the CO and CO_2 occurs in this system.

The determined value for atom percentage excess C^{13} in the CO_2 of the experimental flask of experiment No. 2 was not corrected for any possible contribution from ordinary respiration since the air control in this experiment was not analyzed for C^{13}. A correction of the same order as in experiment No. 1 would lower very little the calculated percentage of CO_2 derived from oxidation of CO (column 6).

The principal source of uncertainty in these two experiments is CO_2-retention in the egg suspensions of the Warburg flasks. As shown in later experiments the egg suspensions may contain considerable amounts of bicarbonate at the beginning of the experiments, despite the normal precautions to keep this at a low value. This unlabelled bicarbonate would presumably form a common pool during the run with bicarbonate derived both from ordinary respiration and from the oxidation of labelled CO. The CO_2 collected in the alkali for analysis would then have been diluted with the unlabelled CO_2 present in the egg suspension at the start of the experiment. Also, some of the labelled CO_2 produced during the experiment would be retained in the suspension at the end of the run. If corrections were made for the above effects, the values calculated in column 6 for CO-oxidation in experiments No. 1 and No. 2 would be higher than those presented. In other words, the value used for atom percentage excess C^{13} to be expected if only CO-oxidation took place would be lower than those listed in column 2. Therefore, the calculated percentages of CO_2 derived from CO-oxidation in these two experiments represent minimum values.

It should be noted that the expected percentages of CO_2 produced from CO by the eggs, as calculated from excess gas-uptake (column 7), also represent minimum values, since they depend on the assumptions that the R.Q. is 1.0, and that there is no inhibition of ordinary respiration by CO in the light. Lindahl (1939) has shown that in 75% CO/O_2 in the light, the eggs of the sea urchin have a lower R.Q. than one would expect, even if one were to account for all the excess gas-uptake as CO oxidation. This could be due to an inhibition of ordinary respiration by CO in the light, which is masked by the utilization of CO. In the present experiments if an R.Q. of 0.67 instead of 1.0 were assumed for the ordinary respiration, as well as the CO-oxidation, then the expected percentages of CO_2 derived from CO-oxidation (column 7) would be 32 and 30 for experiments No. 1 and No. 2, respectively.

In experiment No. 3 the bicarbonate in the egg suspension, as well as that in the alkali well, was collected for analysis of C^{13} content in the mass spectrometer. A control flask of egg suspension, into which acid was tipped at the time of the first reading of the manometers, provided a measure of unlabelled CO_2 originally present. The retained, as well as the respired, CO_2 was determined before transfer to the reaction vessel of the mass spectrometer, as described in Materials and Methods. The total amounts of CO_2 (375 mm^3. in experimental and 384 mm^3. in control flask) were diluted with 0.5 ml. of carrier 0.04 *M* $NaHCO_3$ (480 mm^3. of CO_2). Initial bicarbonate content of the CO/O_2 blank amounted to 160 mm^3. The corresponding dilution factors applied to the mass spectrometer data were therefore $(375 + 480)/(375 - 160)$ and $(384 + 480)/384$ for experimental and control flasks, respectively. The figures entered in columns 3 and 4 of Table II are corrected for the dilution factor.

The value of 18 per cent for the CO_2 derived from CO-oxidation in this experiment is then not subject to uncertainties of retention and can be considered to represent reasonably closely the extent of CO-oxidation occurring in the dark. Since there is about 3% inhibition of gas-uptake (Table I) in this experiment and since 27% ($\frac{3}{2}$ of 18%) of the gas-uptake represents CO-oxidation, then there is 29% inhibition $(100 - 97\ (0.73))$ of the ordinary respiration by the CO in the dark.

DISCUSSION

The results show that eggs of *Urechis* can oxidize carbon monoxide.This occurs both in the light and in the dark. The amount of carbon monoxide that is oxidized in the light can account for all of the excess gas-uptake that occurs in a $CO–O_2$ mixture. In the dark the percentage of CO_2 derived from CO-oxidation is somewhat less than in the light, according to the present data. It should be noted again that the values obtained for oxidation of CO in the light are probably minimal. In other words, there may be a small amount of inhibition of the "ordinary" respiration in the light which is obscured by the oxidation of CO.

It is possible that in the dark CO may be inhibiting, to some extent, its own oxidation. Clark, Stannard and Fenn (1950) found that sodium azide and hydroxylamine completely blocked the oxidation of CO by skeletal muscle, as measured both by manometric and isotope techniques.

Information available from the literature and from the present experiments does not permit identification of the enzymatic system(s) involved in the oxidation of CO. It seems likely that a haem compound is involved because of the known affinity of CO for the Fe^{++} of such substances. Also, it may well go through cytochrome oxidase. However, tests of cytochrome oxidase preparations from *Urechis* and sea urchin eggs (to be reported later) gave no oxidation of CO.

In certain bacteria CO can serve as the sole carbon source (*cf.* van Niel, 1954). Fixation of CO has been demonstrated in barley leaves (Krall and Tolbert, 1957), in which the labelled carbon appears initially in serine and choline. This fixation occurs in both light and dark but the rate is much higher in the light. The possibility of fixation of CO has not, as yet, been examined in animals, but it does seem likely that some of the CO_2 produced by its oxidation would be assimilated.

As previously reported (Rothschild and Tyler, 1958) and as noted here, the development of the eggs was not significantly accelerated or retarded in the $CO–O_2$

mixtures in the light. It might appear, then, that the energy released by the oxidation of the CO is not put to useful developmental work in this system. However, it should be noted that the CO-oxidation would provide much less energy per mole of carbon than the oxidation of the ordinary substrates of the cell. So, even if the energy were utilized, the increase in developmental rate might be too small to be readily detected under the present conditions in which roughly 25 per cent of the respiration is attributed to oxidation of CO. Furthermore, as indicated above, the figure of 25 per cent is a minimum value. Some inhibition of ordinary respiration could be occurring in the light. If, for example, the inhibition amounted to 25 per cent and if it is assumed that oxidation of CO supplies half as much energy per mole of carbon as does the ordinary respiration, then the total rate of energy supply would be the same for eggs in 80% CO/O_2 in the light as for eggs respiring in air. It is then possible that the energy released by oxidation of CO is utilized by the cell for developmental work.

SUMMARY

1. The fertilized eggs of *Urechis caupo* have been found to oxidize CO to CO_2 both in the light and in the dark. This has been shown by the use of C^{13}-labelled CO. In the light there is a previously described increase in gas-uptake in 80% CO/O_2 as compared with air. All of this excess gas-uptake can be attributed to the oxidation of CO.

2. In the dark the percentage of respiratory CO_2 derived from CO is less than in the light. If the oxidation of CO is subtracted from the total gas uptake, the "ordinary" respiration is shown to be inhibited about 29% in the dark by 80% CO/O_2.

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THE SALT GLAND OF THE HERRING GULL[1]

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The long known fact that the nasal gland is conspicuously larger in marine birds than in terrestrial species has recently been given a functional explanation. It has been found that in birds taking their food from the ocean the nasal gland is developed into an organ whose main function is the secretion of salt. We have, therefore, in our publications on the function of this gland, referred to it as the "salt gland." After large salt intake, due to ingestion of sea water or marine invertebrate organisms, the salt gland assists the kidney in the excretion of excess of sodium chloride. In some marine birds the gland is more important than the kidney in the elimination of salt from the organism (Schmidt-Nielsen and Sladen, 1958; Schmidt-Nielsen and Fänge, 1958b).

The anatomy of the avian nasal gland in a large number of birds, both terrestrial and marine, was described in a monograph by Technau (1936). Although Technau mainly dealt with the gross anatomy of the gland he also made histological observations. Other microscopical observations have been made by Marples (1932) and Mihalik (1932), and the embryology has been studied by Grewe (1951).

The discovery of the osmoregulatory importance of the salt gland of marine birds made it necessary to re-investigate its histology in the light of the present knowledge of its function.

MATERIALS AND METHODS

The material consisted of young specimens of the herring gull (*Larus argentatus*) caught at the Atlantic coast at Beaufort, North Carolina, and at Mount Desert Island, Maine.

For histological examination glands were fixed in Bouin's fluid, and paraffin sections were stained in azan (Romeis, 1924) or haematoxylin-eosin.

The main structure of the arterial supply to the gland was studied by injection of methacrylate plastic into the carotid arteries, followed by maceration of the tissues with KOH. The detailed vascularization was studied in preparations injected with India ink through the carotids, fixed in Bouin's fluid, and subsequently cleared in benzyl benzoate. The glandular duct system was studied by injection of India ink or methacrylate plastic into the lateral duct opening. Paraffin sections were prepared of some of the India ink-injected specimens.

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Gross anatomy

In the gull the large, paired salt gland is situated on the top of the skull in the supraorbital grooves of the frontal bone (Fig. 1). Strictly speaking each gland consists of two parts, as seen from the fact that there are two ducts on each side of the head leading forwards into the beak (Technau, 1936). However, the two parts of the gland have a similar structure and are joined so closely together that they can be considered as one functional unit and may be regarded as one gland. Thus, the glands are flat and crescent shaped, and two ducts pass from the anterior end of each to the anterior nasal cavity (vestibulum). On the upper side the gland is covered by a thin, tough connective tissue membrane. The anterior part of it extends somewhat laterally from the margin of the frontal bone and forms part of the roof of the orbit. Blood vessels and nerves pass from the orbit into the gland through holes in the frontal bone.

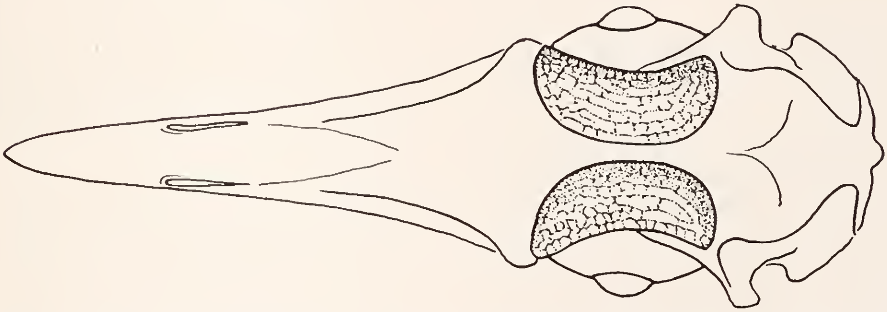


Figure 1. Skull of the herring gull from above, showing the position of the salt gland.

The two ducts on each side of the head take their origin from the lower side of the anterior part of the gland and run close together into the beak, where they open at the posterior end of the vestibular concha (Marples, 1932; Technau 1936). The lateral duct opens on the lower median side of the vestibular concha (pre-concha) while the median duct has its opening on the nasal septum close to the transverse fold separating the anterior nasal cavity (vestibulum) from the upper nasal cavity. The openings of the ducts can be found if a longitudinal incision is made in the palate somewhat lateral to the midline, and it is then possible to cannulate the lateral duct opening for the collection of secretion in living birds (Fänge, Schmidt-Nielsen and Robinson, 1958), or for injection of fluids into the duct. For some reason our attempts to cannulate the median duct were unsuccessful.

Marples (1932) found in *Larus ridibundus* (black-headed gull) that the ducts are formed at an early embryonic stage as outgrowths from the nasal cavity. Later the ducts branch above the frontal bone, forming the glandular tissue. Corresponding to the branches of the embryonic ducts, the gland of the adult is composed of tubes or lobes, giving it a characteristic surface structure (Fig. 2). Most of the gland consists of long lobes, some of which stretch along the whole length of the gland. In the gland of *Larus argentatus* about 15 such longitudinal lobes can be seen in a transversal section. In addition to these longitudinal lobes there are shorter lobes oriented in various directions.

In our material the combined weight of the two salt glands varied from 700 to 900 mg. The weight of the animals was 700–1000 grams (young specimens). Technau (1936) found in the herring gull a gland weight (probably unilateral), of 555 mg., but in the related common gull, *L. canus*, 150 mg., and in the black-headed gull, *L. ridibundus*, only 50 mg. Of these three gulls, the herring gull is the most salt water-bound species while the black-headed gull is, to a large extent, associated with fresh water. Thus, there is a good correlation between the size of the salt gland and the habitat of the different gull species (Schildmacher, 1932).

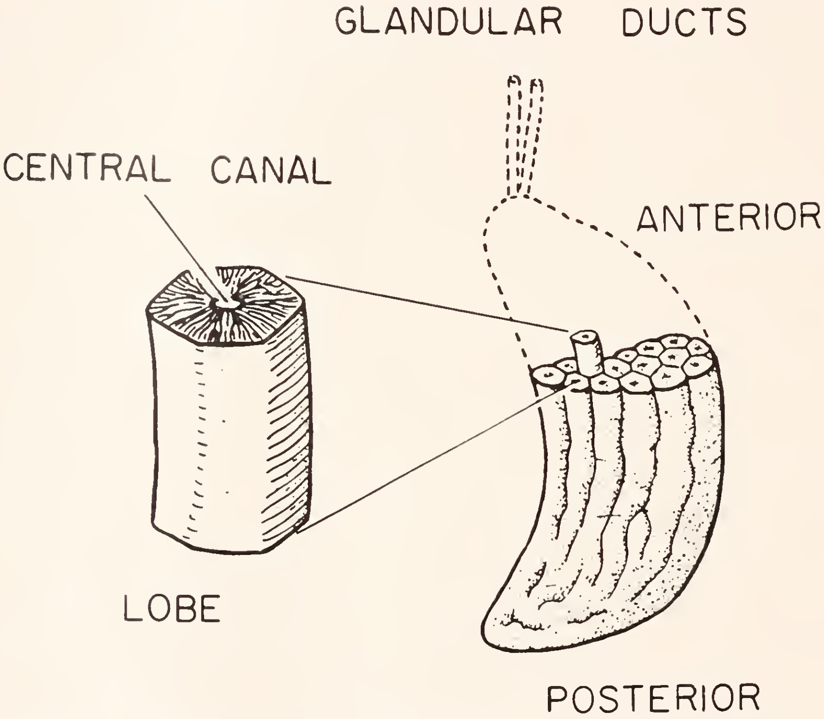
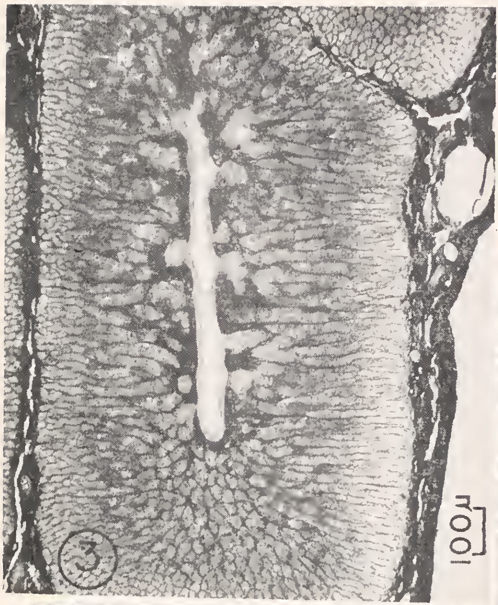
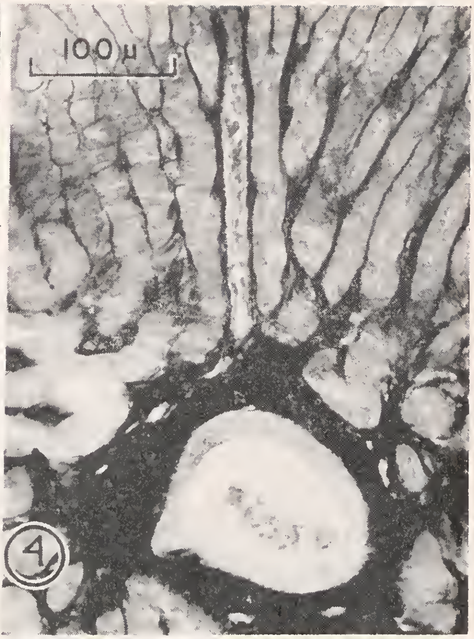


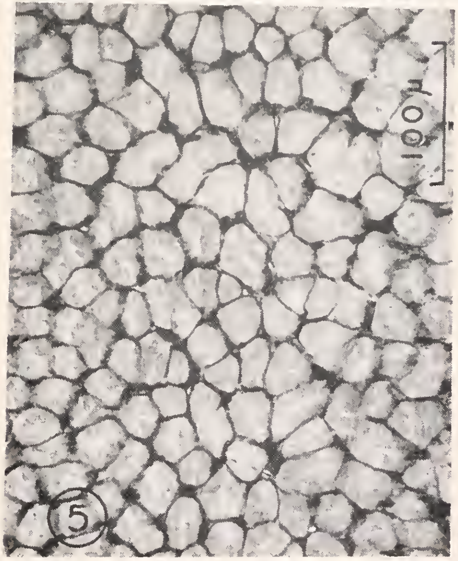
FIGURE 2. Diagram showing the gross structure of the salt gland (left side).

Microscopic structure

In each lobe there is a central canal (Fig. 2) which connects with the lumen of one of the two main ducts from the gland. Branching tubular glands radiate out from this central canal which is surrounded by a rather voluminous connective tissue mass (Figs. 3, 4). Close to the central canal, where the gland tubules have not yet branched extensively, the tubules are round in transverse section and separated by the connective tissue. In the periphery of the lobe the tubules are closely packed together and run parallel to each other, separated by very delicate connective tissue membranes and blood capillaries. In tangential sections through







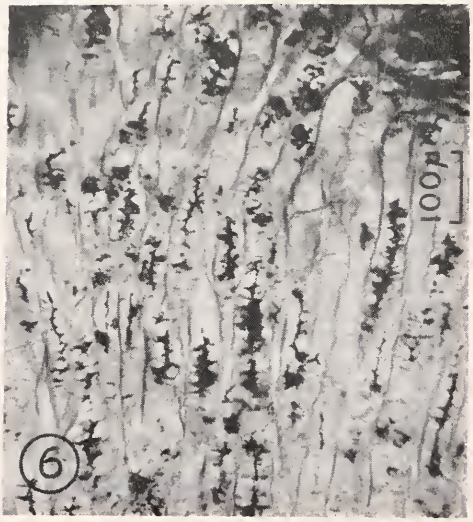


FIGURE 3. Longitudinal section through a lobe. Note the difference in stainability between the outer and the inner zone of tubules. The blue-stained connective tissue is dark, due to use of a yellow filter when taking the microphotograph. (Bouin's fluid, azan.)

FIGURE 4. Transverse section through the central part of a lobe. An artery (vertical in the figure) passes into the connective tissue around the central canal. (Bouin's fluid, azan, yellow filter.)

FIGURE 5. Tangential section through a lobe half-way between the surface of the lobe and the central canal. The capillaries between the tubules are partly filled with blood. (Bouin's fluid, azan, yellow filter.)

FIGURE 6. Transverse section through a lobe halfway between the surface and the central canal. India ink was injected into the lateral duct of the gland before fixation. (Bouin's fluid, azan.)

the peripheral parts of a lobe the cross-sectioned tubules have a polygonal outline and form a honeycomb-like pattern (Fig. 5).

The tubules branch 4–6 times forming different "generations" or "orders" of tubules. In the center of the lobe, close to the central canal, the tubules are thick and consist of cylindrical epithelial cells with the approximate dimensions 6 μ × 15–20 μ. In the periphery of the lobe the diameter of the tubules is smaller, and here the size of the cell is 6–9 μ × 6–9 μ. The cell nuclei are about the same size in the central and the peripheral tubules. Thus, the amount of cytoplasm in relation to the nuclear volume is largest in the central portion of the tubules, possibly indicating that these gland cells carry out more work than those of the peripheral portions.

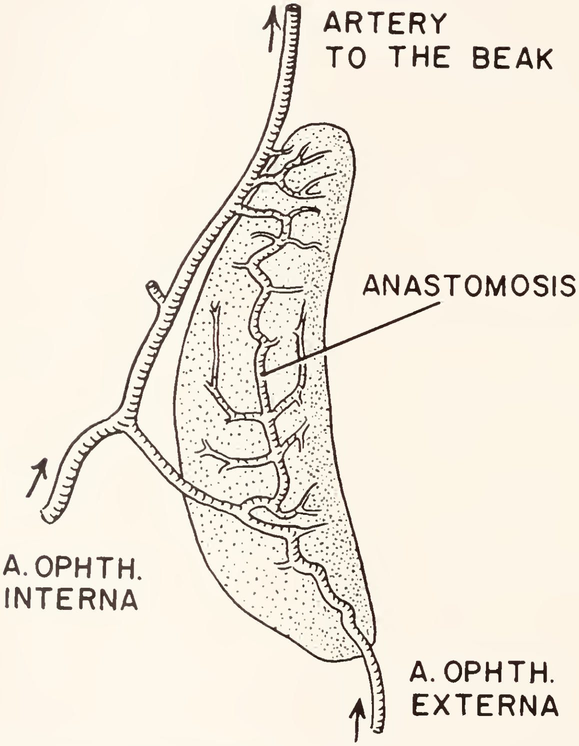


FIGURE 7. The arterial supply of the salt gland. The sketch shows the left gland from below. Drawn from a methacrylate plastic cast of the vascular system.

The cytoplasm of the cells has a lamellated or striated appearance. The striation is not limited to a striated border, but extends through the cells from the lumen to the periphery, where the cells are in contact with blood capillaries. In sections from specimens in which India ink had been injected into the lateral duct, the lumen of the tubules had an irregular shape, indicating the presence of secretory intra- or intercellular canaliculi (Fig. 6).

The cytoplasm takes a reddish colour in azan stain. In the most peripheral part of the tubules the cytoplasm is less heavily stained than in the central tubules (Fig. 3).

The central canal consists of 2–4 layers of cuboidal epithelium. The two main ducts passing to the anterior nasal cavity also consist of a multi-layered epithelium. In some preparations the boundaries between the epithelial cells, especially those of the central canal, had a vacuolated appearance which gave the illusion of a system of intercellular canals. This, however, could be a fixation artefact due to shrinkage of the cells. The two main ducts are surrounded by the same connective tissue which surrounds the accompanying blood vessels and nerve and have no connective tissue of their own. No smooth muscle cells could be found in the walls of the ducts. Neither was it possible to detect any smooth muscle in the gland except that of the arteries supplying the lobes. Neither the connective tissue of the upper side of the gland, the interlobular connective tissue mass, the connective tissue membranes around the tubules, nor the central connective tissue mass around the central canal contains any smooth muscle.

Vascularization

The blood supply of the nasal glands of the duck has been described by Marples (1932) and earlier authors (Gadow, 1891). According to our observations in *Larus argentatus* the main arterial supply comes from the arteria ophthalmica interna. The vessel penetrates the wall of the orbit above the optic nerve and, passing upwards along the median wall of the orbit, it divides into two branches to the salt gland. The anterior branch gives off several small arteries to the gland and then continues into the beak (Fig. 7). The posterior branch supplies the posterior part of the glands. Anastomosing with this branch another artery from the posterior wall of the orbit also gives blood to the gland. This artery probably corresponds to the arteria ophthalmica externa described by previous authors (Gadow, 1891; Slonaker, 1918). Both the arteria ophthalmica interna and the arteria ophthalmica externa are branches of the arteria carotis interna.

The arteries reaching the salt glands are among the largest arteries in the head of the gull. The arrangement of the arteries is such that, in spite of the rich blood supply, the blood could probably bypass the gland via the arterial arch formed by the anastomosis between the anterior and posterior branch of the arteria ophthalmica interna (Fig. 7). This arrangement may permit a large reduction in glandular blood flow without reducing the blood flow to the upper beak when the glands are not functioning. The control of the blood flow through the glands may be exerted by contractile arterioles in the glands.

The veins from the salt glands follow the arteries in their main courses (Marples, 1932).

Microscopic distribution of blood vessels

The connective tissue between the individual gland lobes contains a large number of branching arteries and veins. At intervals the arteries give off branches which pass into the lobes. These arteries pass straight through the gland tissue between the tubules towards the central canal without branching (Figs. 4, 8), but after reaching the central connective tissue mass they break up into numerous capillaries. These capillaries, which have frequent branchings and anastomoses, run radially out towards the surface of the lobes. In their main course the capillaries are parallel to the tubules. Tubules and capillaries form a regular pattern in sections cut tangentially through a lobe (see Fig. 5). The tubules are polygonal in shape and the capillaries are situated at the corners of the polygons, each tubule being surrounded by 5–7 capillaries. The regularity of the arrangement reminds of the rete mirabile of the fish swimbladder, or the regular arrangement of tubules and blood vessels in the medulla of the mammalian kidney. At the surface of the lobe the capillaries leave the tubules and pass over into a venous plexus drained by veins in the interlobular connective tissue (Fig. 8). No veins were observed within the lobes. No lymph vessels could be observed in the glands, but as they may be difficult to detect in histological sections, we hesitate to claim that there are none in the salt gland. A diagrammatic picture of the blood flow in the gland is shown in Figure 9.

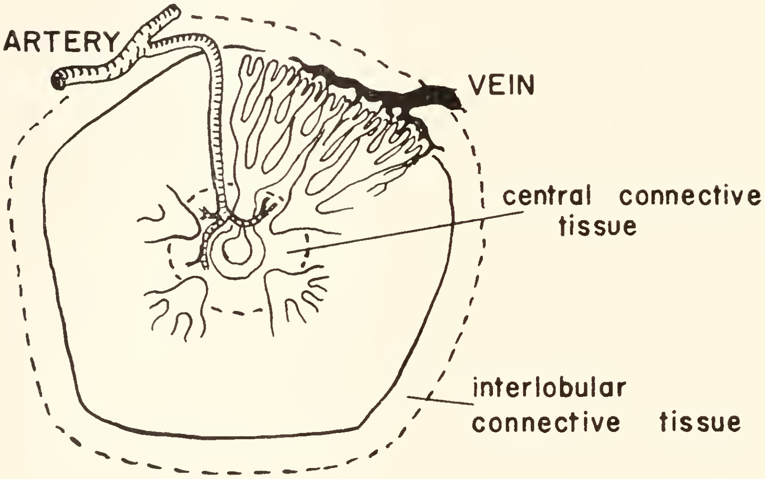


FIGURE 8. Diagram of a transverse section through a lobe of the salt gland.

Innervation

The nasal gland of birds has been reported to be innervated from a parasympathetic ganglion in the anterior part of the orbit (Cords, 1904; Webb, 1957). The ganglion has connections with different cranial nerves and with the sympathetic system (Cords, 1904). The nerve supply of the salt gland in the herring gull will be described in another publication which will also deal with the physiological responses of the gland to various kinds of stimulation (Fänge, Schmidt-Nielsen and Robinson, 1958).

Other bird species

The presence of salt glands has been demonstrated in birds of five different orders (Schmidt-Nielsen and Fänge, 1958a). We have undertaken some pre-

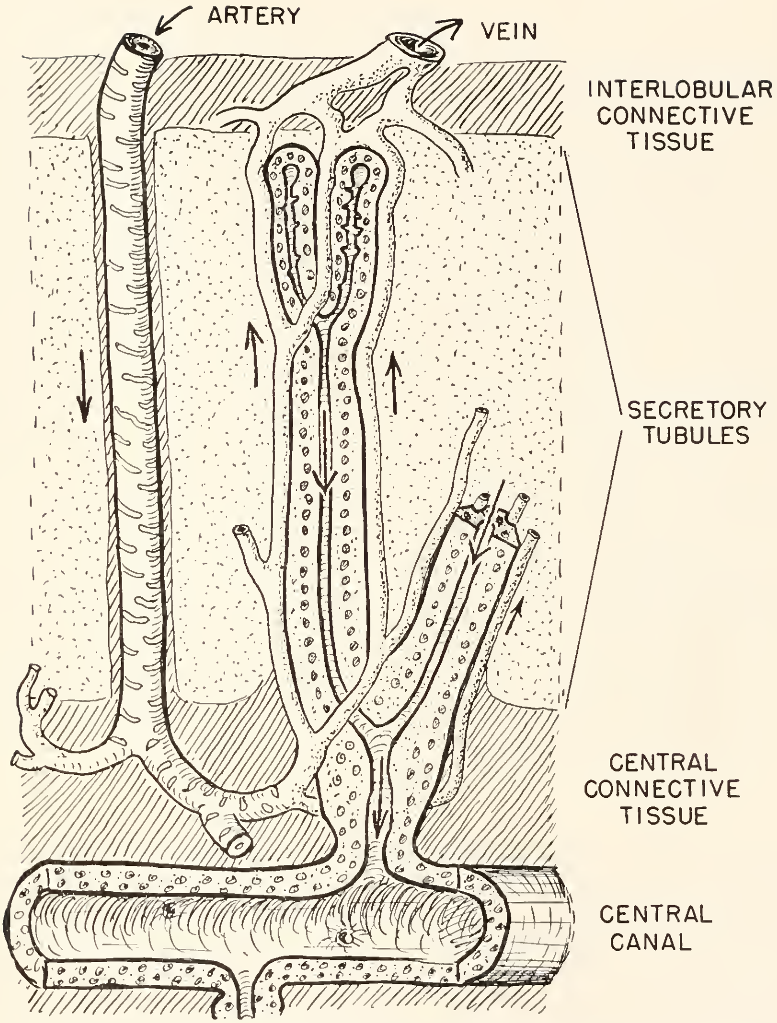

ARTERY
VEIN
INTERLOBULAR CONNECTIVE TISSUE
SECRETORY TUBULES
CENTRAL CONNECTIVE TISSUE
CENTRAL CANAL

FIGURE 9. Diagram of the circulation showing the opposing directions of the flow in the gland tubules and in the capillaries. The tubules branch repeatedly, but for simplicity only two ramifications are pictured.

liminary histological studies of the salt glands of pelican (*Pelecanus*), cormorant (*Phalacrocorax*), eider duck (*Somateria*), petrel (*Oceanodroma*), etc. In these birds the glands have essentially the same histological structure as in the gull, and consists of lobes with tubular glands radially arranged around a central canal. In the pelican and the cormorant the lobes are not tubiform as in the gull, but rather short and of a rounded shape. In the connective tissue of the salt glands of many birds black pigment cells occur.

Discussion

The salt gland of marine birds has a very characteristic structure consisting of closely packed secretory tubules with blood vessels between them. The tubules radiate from a central canal. In terrestrial birds, where the nasal glands have no salt excretory function, the glands contain only a few tubules or have sac-shaped diverticula instead of tubules (Marples, 1932). The strictly parallel arrangement of closely packed, glandular tubules may be necessary for the osmotic work performed by the gland. It is probable that the manner of distribution of the blood capillaries within the gland tissue is also of importance in this respect. It may be noted that the arrangement of blood vessels within the lobe is such that the capillary blood flows in a direction opposite to that of the secreted fluid. The functional significance of this counter-current flow in the salt gland is not clear. The counter-current principle, although manifested in a different way, seems to play an important role in the production of a concentrated urine in the kidney of mammals and birds (Hargitay and Kuhn, 1951). Although the structure of the salt gland in marine birds and of the mammalian kidney otherwise are entirely different, it is striking that a counter-current flow is found in both these organs, which in higher vertebrates are the only ones known to produce a highly hypertonic secretion.

The counter-current flow in the salt gland cannot, as such, explain the large osmotic work performed by the gland. Active ionic transport can be assumed to be the fundamental cellular process responsible for the osmotic work. The striated or lamellated appearance of the cytoplasm of the gland cells and the presence of secretory canaliculi indicate a highly specialized transport function of the cytoplasm. A more detailed study of the microscopic and electron microscopic structure of the cytoplasm of the avian salt gland cells is in progress.

Summary

1. The salt gland of the herring gull (*Larus argentatus*) is a large, paired gland on top of the skull. On each side two ducts lead to the anterior nasal cavity. When the gland is secreting, its discharge comes out through the nares and drips off from the tip of the beak.

2. The gland has long, tubular lobes, each with a central canal. Tubulous glands radiate from the central canal. The gland cells have a striated or lamellated cytoplasm, and seem to have secretory canaliculi.

3. The blood supply is mainly from arteria ophthalmica interna. Within the gland the capillary blood flow is in a direction opposite to that of the secreted fluid. The innervation of the gland is from a ganglion of predominantly parasympathetic nature.

4. The salt glands of other marine birds have the same characteristic structure with the secreting tubules radiating out from a central canal.

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THE SWIMBLADDER OF THE TOADFISH (OPSANUS TAU L.)

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The swimbladder of the toadfish (*Opsanus tau* L.) offers a particularly favorable object for the experimental study of gas secretion. To provide a basis for physiological studies we describe here the structure of the swimbladder, its gas gland and its vascular supply. In addition, some physiological observations are presented. Further physiological studies of this species are reported elsewhere (Wittenberg, 1958).

Brief anatomical descriptions of the swimbladder of the toadfish are found in Tower (1908) and Rauther (1945). Greene (1924a) has studied a related species, *Porichthys*. Tracy (1911) presents some embryological and histological data. Tracy observed that the posterior chamber of the embryonic toadfish develops from the pneumatic duct, which secondarily loses its connection with the gut.

MATERIAL AND METHODS

Animals: Toadfish caught at Woods Hole were maintained in a shallow live car for several months before they were used.

Histological: After fixation in Bouin's fluid, histological sections were made and stained with azan (Romeis, 1948) or haematoxylin and eosin. The blood vessels were studied by injection of India ink into the coeliac artery. The injected specimens were fixed in Bouin's fluid and later cleared in benzyl benzoate.

Gas analyses: These were by the method of Scholander *et al.* (1955).

RESULTS

The swimbladder gases

In contrast to the majority of shallow-living marine fishes, the toadfish normally maintains a very high proportion of oxygen in the swimbladder gases. The oxygen ranges from 40 to 80 per cent and in most animals is about 50 per cent of the total gas. Similar high oxygen concentrations (maximum 88 per cent) have previously been observed in a related species, *Porichthys* (Greene, 1924b).

When forced experimentally to renew repeatedly the gaseous contents of the bladder, the toadfish is able to maintain the secretion of gas undiminished in rate and oxygen content. Thus in one experimental series the swimbladders of three animals were emptied every 24 hours for six days. During this time, each animal

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[2] This investigation was supported by a Senior Research Fellowship (S.F. 57) from the Public Health Service, and by a research grant from The National Science Foundation.

secreted a volume of gas equivalent to six times the volume of the swimbladder. At the end of the six-day period, the rate of secretion and the composition of the secreted gas remained unchanged. The newly secreted gas is characterized by an extraordinarily high proportion of oxygen which averages 90 per cent and may be as high as 96 per cent of the total gas. The proportion of carbon dioxide is low, about 4 per cent (Wittenberg, unpublished data). The ratio, argon to nitrogen, in the secreted gas is very high, 2.4×10^{-2} to 2.6×10^{-2}, and approaches the maximum which can be achieved by a mechanism of inert gas secretion proposed elsewhere (Wittenberg, 1958). These properties combine to indicate a very powerful development of oxygen transport in the gas gland of the toadfish, making this an animal of choice for experimental studies concerning oxygen transport.

The principal layers of the swimbladder wall

The external appearance of the swimbladder is shown in Figure 1. It is of the euphysoclist type (Rauther, 1922; Fänge, 1953). The wall may be described as formed of three layers, conveniently called tunica externa, submucosa and mucosa. The tunica externa is a tough, somewhat rigid external connective tissue capsule. Laterally this layer includes the sound-producing striated muscle masses (Figs. 1 and 2; compare with Rauther, 1945).

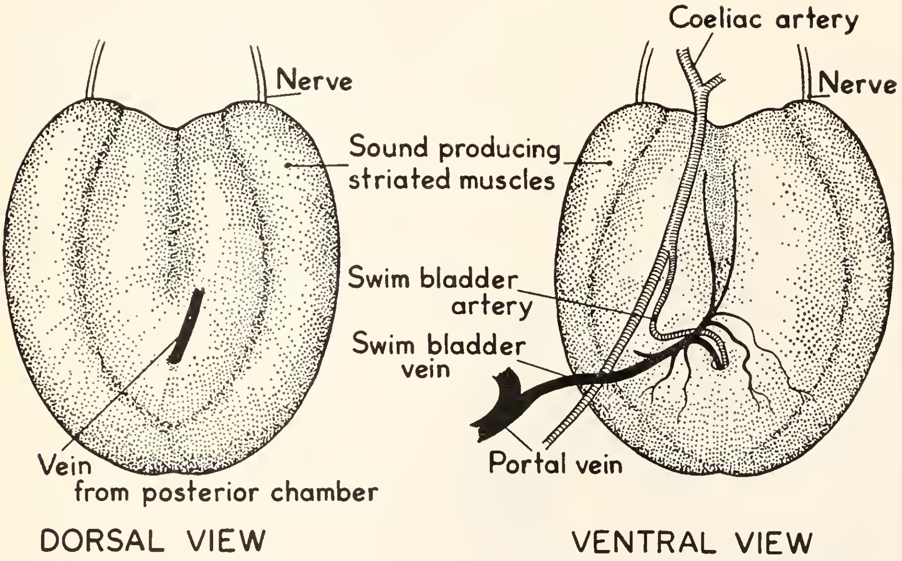

FIGURE 1. External view of the swimbladder of the toadfish seen in dorsal and ventral view. The nerve shown in the picture is the motor nerve to the striated sound-producing muscle. According to Tracy (1911) it is a branch of the first spinal nerve.

The submucosa consists of very loose fibrous connective tissue which allows a limited movement of the mucosa relative to the tunica externa. In fresh specimens it is possible to take advantage of the loose consistency of the submucosa to dissect away the tunica externa, including the striated muscle masses. The mucosa is then revealed as a transparent, richly vascularized, sac composed of two chambers separated by a deep transverse constriction, the diaphragm (Fig. 2). The lumina of the two chambers communicate by a hole in the diaphragm (Fig. 2).

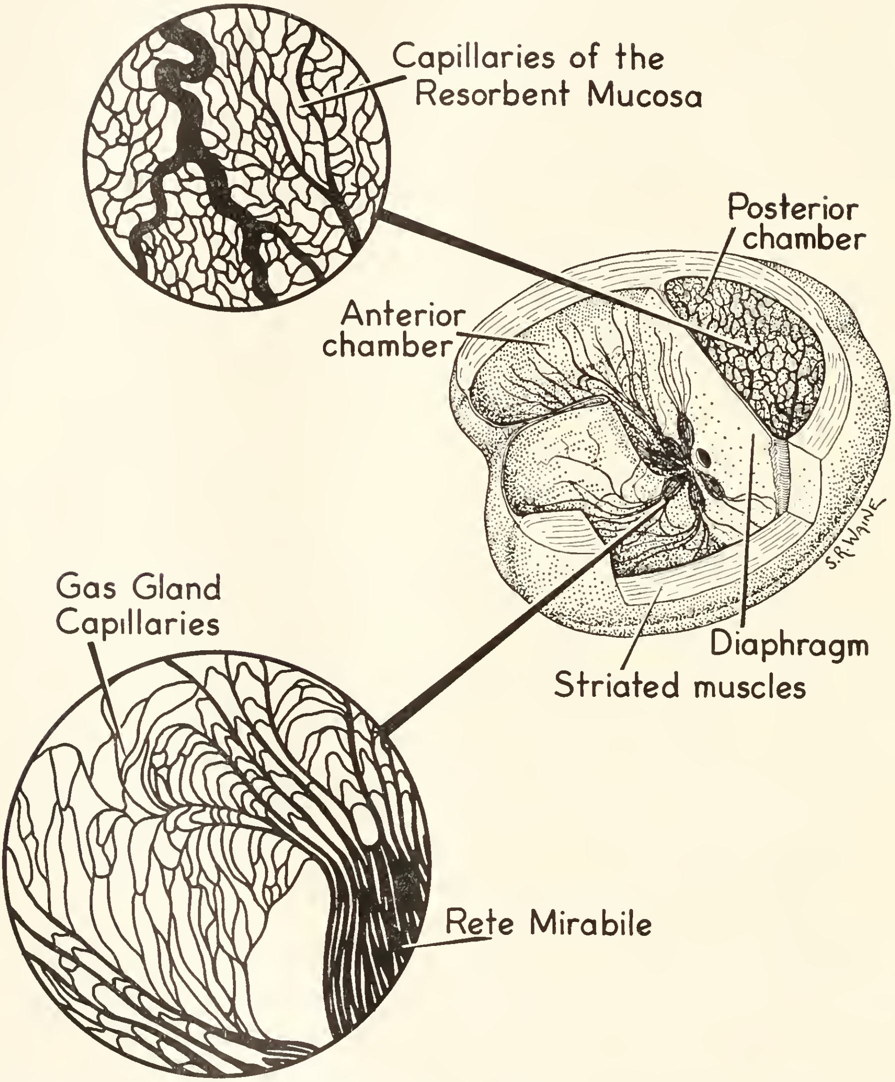
Capillaries of the Resorbent Mucosa
Posterior chamber
Anterior chamber
S.R. WAINE
Gas Gland Capillaries
Diaphragm
Striated muscles
Rete Mirabile

FIGURE 2. The swimbladder opened dorsally. Portions of the secretory mucosa and the resorbent mucosa are shown in higher magnification, in order to demonstrate the typical appearance of the blood vessels.

The anterior chamber, gas gland and retia mirabilia

The gas gland forms the epithelial lining of the floor of the anterior chamber and to a lesser extent it is developed on the anterior face of the diaphragm. Peripherally the gas gland is continuous with the cuboidal, apparently non-glandular, epithelium of the roof of the anterior chamber. The gas gland is most strongly developed and heavily folded within a few millimeters of the retia mirabilia (Fig. 3). At a distance from the retia the degree of folding dwindles rapidly and the glandular cells become smaller. The glandular epithelium is everywhere only one cell thick. The cells are columnar with a dense cytoplasm stained red by azan. An interesting feature of the gas gland cells is the position of the cell nuclei (Fig. 3). These are situated near the secretory lumen and not adjacent to the basal blood vessel as in most gland cells. This peculiar position of the nuclei has been noted by Woodland (1911) in the gas gland of the eel (*Anguilla*) and other species, but in the toadfish the nuclei are situated far more apically than in any of the fish studied by Woodland.

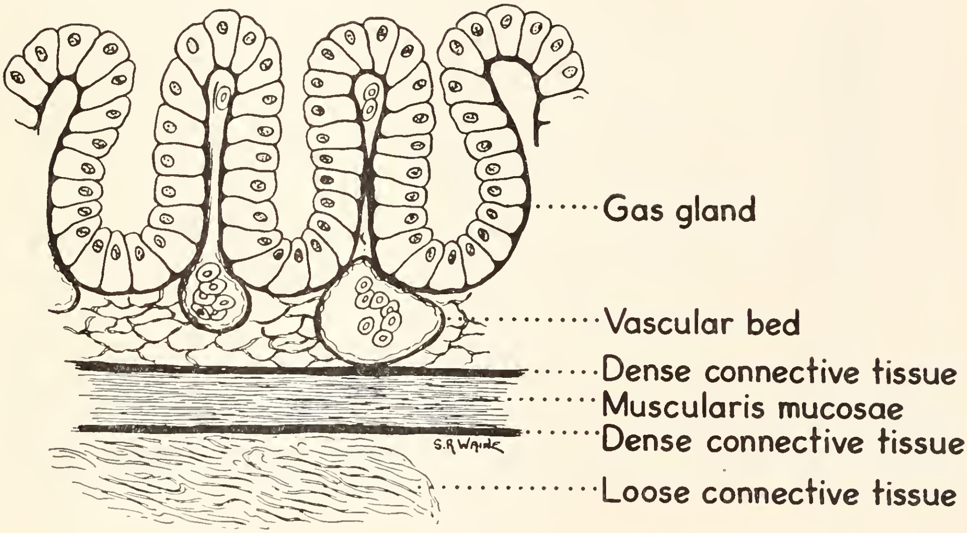

FIGURE 3. Partly diagrammatic drawing of a section through the secretory mucosa. Blood vessels are found within the folds of the secretory epithelium.

The structure of the retia mirabilia is essentially of the type described for the eel by Woodland (1911). There are 6–8 distinct retia ("red bodies") situated in the submucosa at the junction of the floor of the anterior chamber and the diaphragm. The capillaries emanating from the retia mirabilia rejoin, to some extent, forming arterioles and venules which go to the gas gland, where they break up into capillaries providing a very rich blood supply to the glandular membrane. Every fold of the membrane contains blood vessels (Fig. 3), and it is probable that each gland cell has access to a blood capillary at its base and is separated from the blood only by a very thin endothelium. Capillary connections are found between arterioles and venules emanating from the same rete as well as between blood vessels emanating from different retia (Fig. 2).

The capillaries of a single rete mirabile were counted in a histological section. A very rough calculation indicated that the total number of capillaries of all the retia mirabilia is 200,000–300,000, which is of the order of magnitude found by Krogh (1929) in the eel.

In the connective tissue surrounding the central parts of the retia mirabilia there are numerous nerves and ganglion cells. The ganglion cells probably give fibers to the gas gland or innervate the muscularis mucosae.

The muscularis mucosae and the diaphragm

In close connection with the inner epithelium of both the anterior and posterior chamber there is a smooth muscle layer, the muscularis mucosae. This is extremely thin in the posterior chamber but well developed in the anterior chamber, especially ventrally in connection with the glandular portion of the epithelium. The muscularis mucosae also makes a large contribution to the diaphragm where it forms a sphincter around the hole. Tower (1908) observed that the position of the diaphragm varies from about one-third of the distance from the posterior end to less than one-sixth of the distance. We have observed the same variations. That these changes of the position of the diaphragm are due to reflex movements of the muscularis mucosae is shown by the following observations: (1) In a specimen in which gas secretion had been stimulated by emptying the bladder three hours earlier, the diaphragm had a posterior position, by which consequence the anterior chamber was enlarged and the posterior chamber diminished. The hole in the diaphragm was closed. (2) In a specimen which suffered from asphyxiation and which in addition had received an injection of adrenaline (0.1 ml., 1:1000), the diaphragm was found in the anterior position and with its hole open. (Asphyxia and adrenaline each stimulate gas resorption.) (3) In individuals, where the hole in the diaphragm was initially closed, application of a small drop of adrenaline solution to the margin of the hole caused this to open to a width of 2–3 mm. It is evident that movements of the muscularis mucosae are among the physiological regulatory mechanisms which control reflexly the function of the secretory chamber (the gas gland) and the resorbent chamber ("the posterior vascular organ").

The blood supply of the swimbladder

The swimbladder receives its blood from a branch of the coeliac artery, the swimbladder artery (Fig. 1). The individual retia of the anterior chamber are supplied by branches from the swimbladder artery. Within each rete the arterial and venous capillaries form the typical counter-current exchange system studied by Woodland (1911), Haldane (1922), Krogh (1929) and Scholander (1954). All the blood to the anterior chamber passes through the retia. The entire venous return from the anterior chamber passes back through the retia and leaves the swimbladder by the swimbladder vein, which joins the portal vein (Fig. 1, ventral view).

The blood supply to the resorbent capillary network (the "posterior vascular organ") of the posterior chamber resembles that of *Fierasfer* (Emery, 1880) and the eel (Mott, 1950a, 1950b) in that the arterial blood is supplied from the swimbladder artery instead of from the intercostal arteries as in most physoclists. The venous return is to the cardinal vein system (Fig. 1, dorsal view).

Discussion

The swimbladder of the toadfish is of the typical euphysoclist type (Rauther, 1922; Fänge, 1953). It shows many similarities, both physiologically and morphologically, with that of the eel.

The swimbladder of the toadfish is apparently specialized for the production of sounds (Tower, 1908), and the tunica externa forms a thick capsule enclosing both the anterior and posterior chambers. Removal of this capsule reveals the homology of the two chambers with corresponding parts of the eel swimbladder (Fig. 4). (For previous descriptions of the swimbladder of the eel see Queckett (1844), Woodland (1911), Rauther (1922), Fänge (1953).) The anterior chamber of the toadfish swimbladder corresponds to the swimbladder *per se* in the eel and the posterior chamber corresponds to the pneumatic duct of the eel. The homology is further substantiated by the embryonic development of the toadfish swimbladder (Tracy, 1911) during which the posterior chamber develops from the embryonic pneumatic duct. The muscularis mucosae of the toadfish and the eel respond to adrenaline in a similar manner; the anterior chamber of the toadfish swimbladder and the swimbladder of the eel both are contracted by adrenaline while the posterior chamber of the toadfish swimbladder and the pneumatic duct of the eel are relaxed (Fänge, 1953).

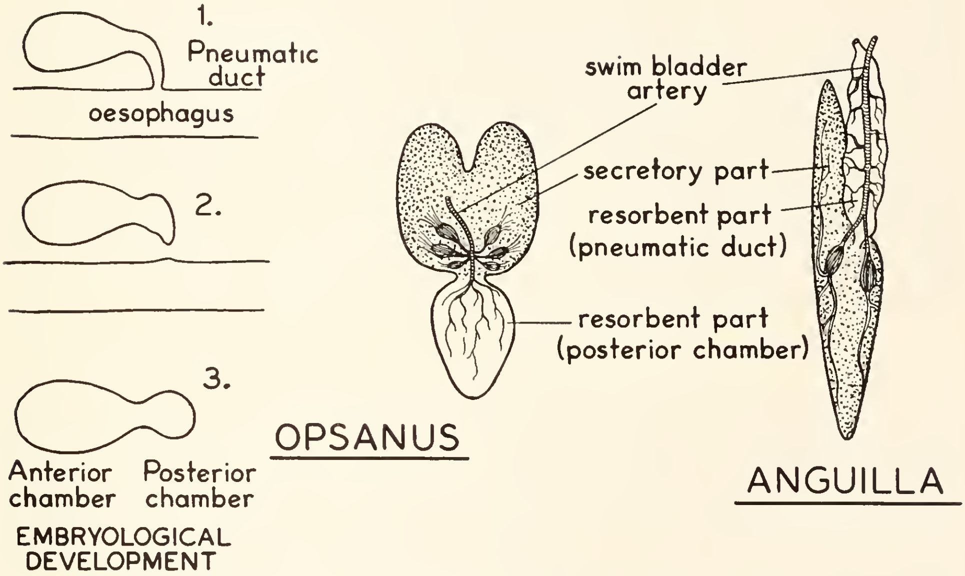
1.
Pneumatic duct
oesophagus
2.
3.
Anterior chamber
Posterior chamber
EMBRYOLOGICAL DEVELOPMENT
swim bladder artery
secretory part
resorbent part (pneumatic duct)
resorbent part (posterior chamber)
OPSANUS
ANGUILLA

FIGURE 4. The swimbladder of the toadfish (*Opsanus tau*) and the eel (*Anguilla anguilla*) illustrating the similarity in general structure. The embryological stages to the left in the figure are redrawn from Tracy (1911). Note the transformation of the embryonic pneumatic duct into the posterior chamber.

Woodland (1911), in his classic description of the gas gland, distinguishes three major types of gas glands: those in which the glandular epithelium is composed of a single layer of cells, those in which the gland is massive, and those in which a primitively single layer of cells is secondarily folded into a massive structure. The toadfish, in common with the eel, belongs to the first category (Woodland, 1911) in which (p. 193) "the glandular epithelium is composed of a single layer of cells which either remains unfolded or is only simply folded. . . ." Microscopically, according to our present observations, the glandular epithelia of the toadfish and the eel swimbladder are scarcely distinguishable in appearance. Woodland further subdivides his first class of gas glands on the basis of the extent of the glandular epithelium and the degree of reunion of the blood vessels. The toadfish belongs to the Syngnathus subdivision, type Ib, in which (p. 195) "the glandular epithelium is restricted in area, not lining the whole of the bladder cavity, and the rete mirabile is contiguous with the gas gland, although a small amount of reunion of the capillaries of the rete may occur before these supply the epithelium."

The gas mixtures secreted into the swimbladders of the toadfish and the eel are markedly similar (Wittenberg, unpublished data; Wittenberg, 1958). The oxygen content is very high, up to 95 per cent, and the carbon dioxide content usually is low, about 4 per cent. The ratio of argon to nitrogen is very high and approaches what may be a theoretical maximal value, 2.6×10^{-2} (Wittenberg, 1958). Both the toadfish and the eel possess highly efficient, powerfully developed, oxygen transporting mechanisms, obviously of very similar nature.

SUMMARY

1. The anatomy of the swimbladder and the gas gland of the toadfish (*Opsanus tau* L.) is described. The swimbladder is of the euphysoclist type. The gas gland is composed of a single cell layer.

2. Both physiologically and morphologically the swimbladder of the toadfish shows strong resemblances to that of the eel (*Anguilla*). The swimbladder normally has a high proportion of oxygen, an unusual feature for fishes living in shallow water.

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SALT AND WATER ANATOMY, CONSTANCY AND REGULATION IN RELATED CRABS FROM MARINE AND TERRESTRIAL HABITATS

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Among the numerous species of brachyuran crabs may be found some which are terrestrial, others which are semi-terrestrial and many which are marine. Within this definitive group of animals of close morphological and taxonomic similarities there is a spectrum of adaptation and the implied regulation of salt and water. This adaptation has succeeded across the marine-terrestrial path of emergence which has proved an insurmountable barrier to all but a few animals. Three species were selected to represent three different degrees of exposure of the animal to the electrolyte and water environment of the sea. The relationship between the electrolyte concentration of the marine environment and that within these animals was investigated to determine the degree of independence and the direction, degree and pathways of electrolyte and water regulation.

The common land crab, *Gecarcinus lateralis* (Frem.), was selected to represent the greatest independence from the marine habitat. It is found in burrows sufficiently high in the banks of beach sand on Nonesuch Island, Bermuda, that these burrows at their deepest do not approach within a meter of the high tide level. Nocturnal and beach scavenger in habit, it is able to go weeks, or even months, without entering the surf. The ghost crab, *Ocypode albicans* (Bosq), selected to represent a somewhat closer relationship to the marine habitat, is found in burrows near and above high tide level on the Delaware ocean beaches. These burrows approach and many have been found to penetrate high tide level with consequent flooding. A nocturnal beach scavenger, it goes into the surf briefly during feeding. The mangrove crab, *Goniopsis cruentatus* (Latr.), almost continuously in water, was selected to represent the closest relationship to the marine habitat. It is found in burrows in the silt and coral basins of mangrove swamps on St. George's Island, Bermuda. It leaves the burrows to seek food at night and may leave the water for brief periods by climbing out on mangrove roots. Seldom found more than a meter above the water and seldom more than two meters from a usable burrow, this sojourn into air appears superficially to be, timewise, a reciprocal of the air-surf relationship shown by ghost crabs.

Gross weight changes are the most obvious indicators where massive inboard or outboard water shifts are suspected, but in box-like animals such as the brachyuran crabs, unilateral water shifts and resulting weight changes may be expected to be of small magnitude. A second indicator might be blood specific gravity shifts resulting from the movement of water into or out of the circulating fluid as a result of electrolyte and osmotic imbalance, this indicated by a greater or smaller fraction of the blood being water.

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[1] With the technical assistance of Sarah C. Flemister.

[2] Contribution No. 243 from the Bermuda Biological Station. Assisted by a Grant-in-Aid from the National Science Foundation through the Bermuda Biological Station.

The absence of gross changes in fresh weight or blood specific gravity does not preclude the possibility of electrolyte and water movement, but instead suggests that this movement results in constant water volumes and electrolyte concentrations. Although chloride ion concentration measurements in the environment, in the blood and in the urine give clear evidence of regulation of concentration in the ghost crab (Flemister and Flemister, 1951), the problem of rate and direction of exchange is difficult, if not impossible, to approach from the chloride ion concentration alone. Rate and direction of exchange may be determined by the use of an ion suitably alike to chloride in its distribution and biological properties in the range of concentrations required for analysis, yet subject to precise measurement apart from chloride. If used in sufficiently small quantities, the resulting environmental, blood and urine concentrations will not interfere with normal chloride movements which would be occurring at the same time, in the same direction, and, presumably, at about the same rate. It is assumed on the basis of an extensive mammalian literature that thiocyanate ions may be used to measure the space into which chloride ions are distributed, this space being profitably termed "extracellular," although some cellular absorption and concentration of both ions are known to occur in some animals. Such concentrations would involve only a limited portion of the data presented here, for this investigation is concerned primarily with the rates of exchange of ions and water between blood and environmental fluids. This is, in effect, a matter of using SCN as a "tagged chloride ion." A second indicator substance is necesary for such a study: a biologically inert non-electrolyte of minimal osmotic effect in the required concentration and of known pattern of movement across certain membranes relative to the movement of water. Inulin clearance is taken to indicate the rate of movement of fluid across the membrane of the antennal gland in a manner which will be called "filtration" for the sake of brevity. The simple filtration of inulin is assumed on the basis of osmotic and hydrostatic measurements on *Carcinus* by Picken (1936), inulin blood: urine ratios of unity found in the lobster, *Homarus*, by Forster and Zia-Walrath (1941) and by Burger (1955, 1957), and the possibility that the work of Maluf (1941), originally thought to indicate inulin secretion in the crayfish, *Cambarus*, may be interpreted differently as pointed out by Martin (1957).

The measurement of chloride, thiocyanate, inulin and water content of environment, blood and urine might be expected to illuminate the constancy of volumes and of electrolyte and water proportions, the direction and rate of movement of electrolytes and of water in the maintenance of the constancy against variation in the proportion of electrolyte to water in the environment.

METHODS

Ghost crabs (*Ocypode albicans*) from the Delaware beaches and land crabs (*Gecarcinus lateralis*) from Nonesuch Island, Bermuda, were kept in individual containers in which there was enough beach sand to allow burrow digging. Mangrove crabs (*Goniopsis cruentatus*) from mangrove swamps on St. George's

Island, Bermuda, were kept in individual containers with fresh sea water about two inches deep and planks on which they could get out of the water. Ghost crabs were fed all the fresh fish they would eat each night and allowed to swim in sea water for about five minutes. The once-a-day feeding and bathing routine paralleled natural conditions and made it possible to keep the animals in good condition for ten days or longer. At Bermuda, where fresh land crabs and mangrove crabs could be obtained more easily and at more frequent intervals, no effort was made to sustain a large number of crabs in the laboratory. Individuals of each species were in good condition after a week or ten days. All crabs were weighed daily and all had been in the laboratory at least twenty-four hours before any work was done with them.

The total water content of the animals was determined as the difference between fresh weight and the constant weight of the minced carcass after drying at 105° C. and cooling. Blood specific gravity of ghost crabs was determined by the method of Jacobsen and Linderstrom-Lang (1940) and blood total water was determined as the difference between fresh and dried weights of 1- to 2-cc. blood samples. None of the crabs used for these determinations were used in any other procedure.

Blood concentrations of thiocyanate and inulin, determined at 30-minute intervals on animals kept in dry containers during the three hours following injection of known amounts of the compounds, were used as the basis for extrapolation to the concentrations which would have been produced by complete and instantaneous distribution. The indicated concentrations were used to calculate the volumes available for SCN and inulin dilution. The variability of these volumes was appraised in relation to the total body water volume in 8 to 12 animals of each species. The blood and urine chloride concentrations and their variability were determined on a similar number of animals in dry containers. This quantitative characterization, the fluid and chloride anatomy, was used as the basis for the demonstration of regulation of electrolyte and water proportions in animals exposed to environmental salt and water stress. The rates of SCN and inulin loss and of SCN absorption were used to determine the rate, direction and pathway of electrolyte and water movement in these stress situations.

One-tenth of a cubic centimeter of blood was drawn from the sinus within the proximal joint of one of the legs, using a No. 27 needle fitted to a clean, dry one-quarter cubic centimeter tuberculin syringe in a holder. No anticoagulant was used. It was found that quick, smooth handling of the blood could effect the measurement and transfer of aliquots before clotting commenced. Aliquots of this blood sample were prepared as blanks for reference setting of the spectrophotometer for SCN and inulin measurements and the determination of control chloride concentrations. Injection of either 0.100 to 0.200 cc. 3% NaSCN (Merck Reagent), 0.100 to 0.200 cc. 5% inulin (Pfanstiehl C. P., re-crystallized), or 0.100 to 0.200 cc. 5% inulin in 3% NaSCN was made deep into the same sinus from a fixed-delivery syringe and needle. Immediately the same volume of the same solution was introduced into a 5-, a 10- and a 25-cc. volumetric flask, made to volume and samples taken from these were analyzed along with the blood samples for the precise determination of the amounts of NaSCN and inulin injected. Slow, deep introduction of injected fluid and careful sampling from deep within the sinus usually prevented fluid or blood loss from the site of puncture. In the few cases where fluid loss or bleeding did occur, the animals were discarded.

At fixed intervals after the injection of SCN and inulin, one-tenth of a cubic centimeter of blood was drawn from the sinus of the proximal joint of one of the legs on the side opposite the injection site. Two 0.500-cc. samples of diluted blood were prepared by transferring 0.040 cc. blood to 3-cc. test tubes, each containing 0.460 cc. distilled water. The transfers were made by separate, clean, dry measuring micropipettes of the Folin type which were flushed into the 0.460 cc. distilled water with repeated rinsing of the pipette lumen with the resulting 0.500 cc. of diluted blood. To one of the samples of diluted blood was added 1.00 cc. 10% CCl_3COOH, the mixture shaken thoroughly, centrifuged, and 1.00 cc. of the supernatant fluid transferred to a Coleman cuvette. Two-tenths of a cubic centimeter 10% $Fe(NO_3)_3 \cdot 9\ H_2O$ in 5% HNO_3 was added with thorough mixing, and the optical density of the resulting $Fe(SCN)_3$ was read immediately at 490 mμ and the SCN concentration calculated. This procedure is a modification of a method introduced by Crandall and Anderson (1934). On the second sample of diluted blood a Somogyi (1930) precipitation of protein was carried out by adding 0.50 cc. 10% $ZnSO_4 \cdot 7\ H_2O$ with thorough mixing and then adding 0.50 cc. 0.5 N NaOH, the mixture mechanically shaken for 30 minutes, centrifuged, and 1.0 cc. of the supernatant transferred to a 9-cc. test tube. Following in principle a method introduced by Young and Raisz (1952), 0.25 cc. 4 N NaOH was added with thorough mixing, the tube closed by a glass marble, and the contents heated in a boiling water bath for 15 minutes. The contents were cooled, and 6.25 cc. anthrone reagent, 0.4% anthrone (Matheson, Coleman and Bell) in 75% H_2SO_4, were added with cooling. The tube was again closed by a glass marble and the contents heated in a 75° C. water bath for 5 minutes, cooled, allowed to stand 30 minutes at room temperature, transferred to a Coleman cuvette, the optical density read at 630 mμ and the inulin concentration calculated.

From the sample remaining in the syringe and needle after the transfer of the two 0.040-cc. portions, blood was drawn to the 1 mark in a Thoma pipette and diluted to the 11 mark with distilled water. The contents were mixed and blown into a small glass cup with repeated rinsing of the pipette lumen. Duplicate 0.020- or 0.100-cc. aliquots of the diluted blood, depending on chloride concentration, were transferred by a micro blood sugar, Folin, pipette to 0.200 cc. distilled water in each of several depressions in a Coors porcelain plate with rinsing of the micropipette lumen with the now doubly diluted blood. Two-tenths of a cubic centimeter of 1 N H_2SO_4 was added. With mechanical stirring, 0.010 N $AgNO_3$ was added in small increments from a Scholander micrometer burette (Scholander *et al.*, 1943) and the potentiometric titration of the chloride ion was accomplished by the method of Cunningham, Kirk and Brooks (1941). Appropriate blanks and the determination of known standards accompanied the measurements of all unknowns.

Urine, collected as described by Flemister and Flemister (1951), was diluted in a Thoma pipette, aliquots taken and chloride, SCN and inulin concentrations determined by the procedures described for blood. Appropriate dilutions were made to hold concentrations within the sensitive range of the methods.

After determination of sea water chloride concentrations by the method described for blood, dilutions were made with distilled water and concentrations were accomplished by evaporation at room temperature to prepare environmental fluids containing 120, 240, 360, 480, 600 and 720 mM. Cl/L. To this series, which was checked for chloride concentration after preparation, was added distilled water, presented in the graphs as 0 mM. Cl/L. Data from animals exposed to air rather than environmental fluids are presented as "dry." Animals were exposed individually to the various environmental fluids. They were placed in glass containers with sufficient volume, 200 to 300 cc., of the fluid to cover their bodies in the resting position. Fluids were renewed every twelve hours. Environmental solutions were prepared for SCN uptake by adding 1.00 or 2.00 cc. 3% NaSCN to each 100 cc. of environmental fluid. The amount added was fixed by the expected rate of absorption in order to keep blood concentrations within reasonable physiological limits and within the sensitive range of the procedures used for measurement.

RESULTS

Fresh weights of land crabs (*Gecarcinus lateralis*), 15 to 45 gm., ghost crabs (*Ocypode albicans*), 20 to 50 gm., and mangrove crabs (*Goniopsis cruentatus*), 20 to 50 gm., were random in distribution with no relation to sex or time of year. Gravid females were not collected. All animals were in the inter-molt period during the time they were in the laboratory. There was no significant weight gain or loss in ghost crabs maintained in the laboratory for as long as three weeks, nor in land crabs and mangrove crabs kept in the laboratory for a week or ten days. All variations in individual weights during captivity were less than 2.8% of the first weight determined soon after capture.

There was no appreciable, consistent change in fresh weight after 72 hours in any of the environmental fluids (120, 240, 360, 480, 600 and 720 mM. Cl/L.) except distilled water (0 mM. Cl/L.). In land crabs and ghost crabs exposed to distilled water for 24 hours and in mangrove crabs exposed for 48 hours, increases in weight never exceeded 4.8% of the fresh weights before the animals were placed in the environmental fluid. In view of the 2.8% variation in fresh weight of crabs in the control group and the difficulty of removing environmental fluid from the gill chambers before weighing in air, these weight changes are not considered significant.

No correlation was found between sex, size, or time of capture and blood specific gravity in 152 recently caught ghost crabs.After the initial determinations, 46 crabs were placed on sand and about 20 in each of the environmental fluids. Among the 46 crabs, after 72 hours on sand, the mean of specific gravity was 1.0442 with a standard error of 0.0009 and the mean for blood total water was 89.2% with a standard error of 0.29. Of about 20 crabs exposed to each of the environmental fluids, only in those surviving 24 hours in distilled water was there a possibly significant change in blood specific gravity, a decrease, with a "P" value between .01 and .05.

1. Water and electrolyte anatomy

Reliable data on volumes of fluid and electrolyte concentrations within the animal are essential to an attempt to determine rates of exchange of water and electrolytes. Measurements of total water content and volume of fluid available for dilution of thiocyanate and inulin on 8 to 12 crabs of each of the three species studied are presented in Table I in terms of per cent of fresh weight. The second

TABLE I

Water and electrolyte anatomy of land crab (Gecarcinus lateralis), ghost crab (Ocypode albicans) and mangrove crab (Goniopsis cruentatus)

	Land Crab	Ghost Crab	Mangrove Crab
Total water			
% fresh weight	66.18	69.93	65.44
S. E.	.52	.70	.63
	64.69–67.66	67.49–72.37	63.46–67.42
SCN space			
% fresh weight	28.52	34.18	30.39
S. E.	.26	.58	.56
	27.79–29.25	32.18–36.18	28.60–32.18
% total water	43.1	48.9	46.4
Inulin space			
% fresh weight	18.80	21.49	19.92
S. E.	.34	.39	.57
	17.84–19.76	20.13–22.85	18.67–21.17
% total water	28.4	30.7	30.4
% SCN space	65.9	62.9	65.5
Blood chloride			
mM. Cl/L.	385	378	422
S. E.	9	7	3
	360–410	354–402	413–431
Urine chloride			
mM. Cl/L.		455	602
S. E.		13	10
		409–501	571–633
Sea water			
mM. Cl/L.	600	480	600

item under each tabulation is the standard error of the mean. The third item in each case is calculated from the standard deviation and is the range within which two-thirds of the data falls, this indicating the variability of the data making up each of the means. Applying the "t" test to the data and taking "P" values less than .01 to indicate significance, .01 to .05 possible significance, and values greater than .05 no significance, the following statements can be made. Although there is a significant, but not marked, difference between the SCN spaces, the volumes of the three compartments in terms of fresh body weight are much alike between land crabs and mangrove crabs. The volumes of the compartments of ghost crabs in the same terms are significantly, and markedly, larger with the exception of the inulin space as compared with that in mangrove crabs. Here the significance of the difference is questionable.

Compared as fractions of total water, the SCN and inulin spaces, presented as the fourth item in the tabulation in each case in Table I, show no significant differences in the three species studied. Therefore, absolute volumes, though showing the differences described in terms of per cent fresh body weight, are of comparable size relative to the total water content of the animal. The average of the means of SCN space is 46.2% and of inulin space 29.8% that of the total water content.

The average of the means of inulin space is 64.7% that of SCN space, fifth item of tabulation, with the fractions for land crabs and mangrove crabs being close together and somewhat larger than for ghost crabs.

Blood chloride concentrations of mangrove crabs are significantly greater than those of land crabs and ghost crabs, in which the concentrations are alike (Table I). A significant difference was found between urine chloride concentrations of ghost crabs and mangrove crabs immediately after capture. The greater concentration in mangrove crabs is to be related to their almost continuous exposure to sea water of high chloride content. Urine samples could not be obtained from land crabs. Within each of the three species, no correlation was found between sex, size, or time of capture and the fluid space available for dilution of SCN or inulin, the total water content, or the concentration of chloride ion in the blood or urine.

II. Evidence of regulation

The effects of exposure to distilled water (0 mM. Cl/L.), 120, 240, 360, 480, 600 and 720 mM. Cl/L. sea water dilutions and concentrations for 24, 48 and 72 hours on blood and urine chloride concentrations in the three species of crabs studied are summarized in Figure 1. The normal blood chloride for each individual animal of each species on sand or in dry containers, plotted as "dry" and placed opposite "1.0" on the ordinate, serves as the basis for representation of the concentrations of chloride ion after exposure to experimental environmental fluids. The mean of the values for normal urine chloride and for blood and urine chloride ion concentration of the same 6 to 10 animals after exposure to experimental fluids, expressed separately as fractions or multiples of the normal blood chloride for the

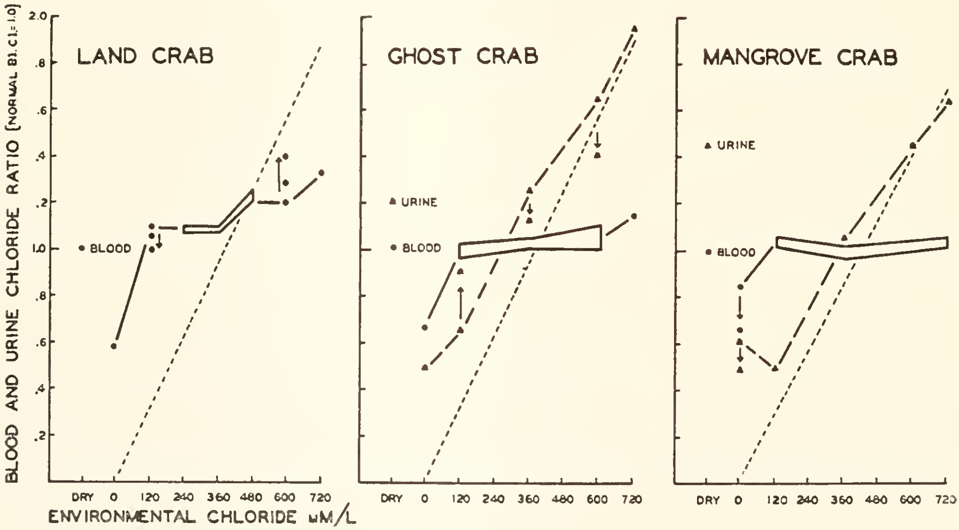

FIGURE 1. Blood and urine chloride concentrations of land crab (*Gecarcinus lateralis*), ghost crab (*Ocypode albicans*) and mangrove crab (*Goniopsis cruentatus*) related to environmental chloride concentrations.

same animal, is plotted for each experimental condition and time interval. Environmental fluid chloride concentrations are represented by a dotted line, blood chloride by a solid line and boxes are used to indicate ranges and concentrations of no statistically significant change during exposure, and urine chloride by a broken line. Additional points and arrows indicate positions and directions of shift of blood and urine chloride curves during exposure from 24 to 48 hours and from 48 to 72 hours. Standard deviations of these individual items of data were relatively small and are not indicated to avoid congestion of the graphs.

Data from land crabs present a picture of effective regulation of blood chloride concentration for 72 hours in mid-range environmental concentrations, though elevated by 10% in 240 and 360 and by 20% in 480 mM. Cl/L. fluids. A breakdown appears before 24 hours in distilled water, soon after 24 hours in 720 mM. Cl/L. with no survivors at 48 hours in either of these environments, and between 48 and 72 hours in 120 and 600 mM. Cl/L. fluids. Blood chloride regulation is somewhat more effective in hypotonic (except 0 mM. Cl/L.) than in hypertonic ranges, but in either, once the breakdown occurs, blood chloride levels approach environmental fluid concentrations. Urine samples could not be obtained from land crabs.

In ghost crabs blood chloride regulation is effective up to 72 hours over the range from 120 to 600 mM. Cl/L. environmental fluids, maintaining concentrations which do not differ significantly from those found in animals on sand. However, regulation fails during the first 24 hours in 0 and 720 mM. Cl/L. fluids. No ghost crab survived to 48 hours in these environmental extremes. Urine chloride concentrations roughly parallel, but are much higher than, environmental levels in all except the high concentrations (600 and 720 mM. Cl/L.). The antennal gland clearly wastes chloride in isotonic and hypotonic environmental situations. Urine chlorides vary little for 48 hours from 120 to 600 mM. Cl/L., but as exposure is prolonged to 72 hours, urine chloride concentrations approach blood chloride levels.

The blood chloride concentrations of mangrove crabs show a striking constancy at near normal levels over the range from 120 to 720 mM. Cl/L. environmental fluids for up to 72 hours. The slight elevations at the extremes fail statistical tests for significance. However, in animals exposed to distilled water, blood chlorides are significantly decreased in 24 hours and markedly so in 48 hours with no survivors at 72 hours. Urine chloride concentrations are very close to environmental levels in 600 and 720 mM. Cl/L. fluids, significantly above environmental levels in 360 and 120 mM. Cl/L. and are markedly elevated in distilled water. In crabs exposed to distilled water, urine chloride concentrations decreased between 24 and 48 hours, but still remained high. Urine chloride levels of animals in all other fluids did not change in 72 hours. These crabs fall short of the classical picture of completely effective regulation only in the breakdown in distilled water and the apparent leakage of chloride in the urine when exposed to 120 and 360 mM. Cl/L. environmental fluids.

III. Evidence of regulatory mechanisms

Data on simultaneous inulin clearance and thiocyanate loss and on absorption of thiocyanate from the experimental environmental fluids into the blood of crabs of the three species studied are summarized in Figure 2. In the clearance and loss curves, SCN and inulin concentrations of blood samples, taken at 1- to 4-hour intervals for the first 12 hours of exposure to environmental fluids (0 to 720 mM. Cl/L.) and then every 8 to 12 hours, are presented as fractions of the concentrations determined immediately prior to exposure and following a post-injection equilibration period of 2 to 4 hours in dry containers. Thiocyanate absorption curves are composed of blood SCN concentrations, determined at similar intervals and expressed as fractions or multiples of environmental SCN levels, in crabs exposed to environmental fluids (0 to 720 mM. Cl/L.) containing small amounts (4 to 7 mM./L.) of NaSCN, after a 2- to 4-hour period in dry containers. Corrections were made in each case for SCN and inulin, or SCN alone, removed from the animal in taking samples. Standard deviations are not represented. They were relatively small except in very low and high concentrations. Sufficient numbers of animals, 4 to 18, were exposed to each of the experimental conditions to achieve statistically significant ("P" value of less than .01) separation by the "t" test. Determinations were made on fresh animals which were discarded at the completion of the 72-hour measurements.

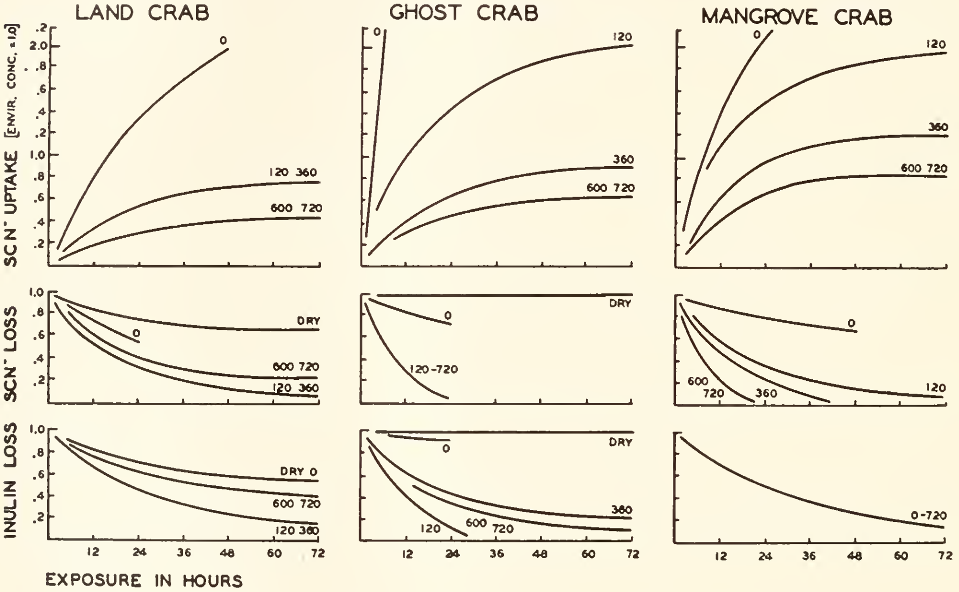

FIGURE 2. Inulin and thiocyanate loss and thiocyanate uptake by land crab (*Gecarcinus lateralis*), ghost crab (*Ocypode albicans*) and mangrove crab (*Goniopsis cruentatus*) exposed to a variety of environmental chloride concentrations.

In land crabs on sand, inulin clearance indicates continuing filtration with the rate of inulin loss slowly and steadily decreasing as blood level falls. Thiocyanate loss, comparable to inulin loss at first, decreases completely as blood concentration levels off, suggesting that SCN is re-absorbed from the fluid which continues to be filtered. The constancy of blood chloride concentration and of blood SCN levels after 24 hours indicates re-absorption of filtered water. In distilled water, inulin is cleared at the same rate as on sand, but SCN blood level falls more rapidly and to about half the beginning value in 24 hours. This is comparable to chloride loss in distilled water and indicates a breakdown in electrolyte regulation not dependent on filtration through the antennal gland. Uninjected animals in 0 mM. Cl/L. fluid containing 4 to 7 mM. SCN/L. absorb SCN at such a rate that despite concurrent loss, which must be assumed, blood levels equal environmental concentrations in 15 hours and are twice as high in 48 hours. Though striking, this absorption results in accumulation of only about 7 mM. SCN/L. during the 24 hours when blood chloride levels fall 162 mM. Cl/L. Land crabs in 120 and 360 mM. Cl/L. fluids clear inulin more rapidly than those on sand or in distilled water, indicating a more rapid turnover, absorption and excretion, of water. In these fluids, SCN loss, more rapid and more complete than inulin clearance, suggests a pathway other than filtration. Blood SCN concentrations of uninjected crabs in these fluids, to which small amounts of SCN were added, are comparable and become steady at 80% of environmental SCN concentration in 48 hours. In all 600 and after 24 hours in 720 mM. Cl/L. fluids, inulin clearances and SCN losses are more rapid and more complete than those on sand or in distilled water, and less rapid and less complete than those in 120 or 360 mM. Cl/L. environments. Again, SCN loss rates, greater than inulin clearance rates, suggest a pathway other than filtration for electrolyte loss. Absorption of SCN from these environmental fluids, containing small amounts of NaSCN, are the same during the first 24 hours and at such a rate that blood SCN levels off at 45% of environmental concentration in 48 hours in animals exposed to 600 mM. Cl/L. fluids. The implication is clearly one of turnover rates reduced from those in hypotonic and near-isotonic fluids.

There appears to be a virtual shutting off of filtration in ghost crabs in the dry situation, judging from the fact that neither inulin, thiocyanate, nor chloride concentrations fall significantly during 72 hours on sand. It was increasingly difficult to get urine samples as exposure to a dry environment was prolonged. This suggests that water gained during brief nightly excursions into the surf is critical for adequate urine formation. The loss of SCN, far exceeding that of inulin in the 24 hours these crabs survived in distilled water, is parallel to the drop in blood chloride level, and is much greater than can be accounted for by a failure in re-absorption after filtration. Absorption of SCN added in small amount to 0 mM. Cl/L. fluids, reaching 3.7 times environmental concentration in 12 hours, 6.2 in 24 and 8.0 in 48, with no survivors at 72 hours, was even more striking than in land crabs, and indicated that 25 mM. SCN/L. was retained in the blood in the 24 hours while 129 mM. Cl/L. was being lost. In 120 mM. Cl/L. fluid, inulin and SCN disappear from the blood at about the same rate with complete removal in 30 hours. In this time sufficient SCN is absorbed from SCN-containing 120 mM. Cl/L. fluid to bring the blood level to 1.5 times the environmental level. This indicates a rapid water and electrolyte turnover with a somewhat excessive retention of ions from the environment which may be compensatory to the loss of chloride due to inadequate re-absorption by the antennal gland. Inulin clearance and SCN loss in 360 mM. Cl/L. environments indicate a much slower filtration and an electrolyte loss, complete in 30 hours, by a route other than filtration. Blood SCN levels in uninjected animals in SCN-containing 360 mM. Cl/L. fluids become constant in 48 hours at about 90% of environmental SCN concentration and at a time when urine chloride level drops from a high toward the environmental, and blood, concentration. In all 600 and for 24 hours in 720 mM. Cl/L. environments, filtration rate is intermediate between those of animals in 120 and 360 mM. Cl/L. fluids and is inadequate to account for SCN loss which is complete in 30 hours. Blood SCN levels, resulting from absorption of SCN added in small amount to these fluids, are the same for the first 24 hours and reach a steady level in animals exposed to 600 mM. Cl/L. fluids at 60% of the environmental concentration in 48 hours.

Inulin clearance rates for mangrove crabs are the same for environmental fluids from distilled water to 720 mM. Cl/L. This indicates a remarkably versatile adjustment of chloride ion concentration in urine, if the regulation of the internal environment is to be maintained, as it apparently is, in contrast to widely different chloride concentrations in the environment. During 48 hours of exposure, SCN loss in distilled water is much less rapid than in the other environmental fluids, less rapid even than inulin clearance, indicating re-absorption of electrolyte after filtration. Thiocyanate absorption from SCN-containing 0 mM. Cl/L. fluid, much more rapid than that from more concentrated environmental fluids, results in blood SCN levels 2.2 times the environmental level in 24 hours, 2.8 in 48 and 3.0 in 72. The 9 mM. SCN retained in the blood at 24 hours does not compare favorably with the blood chloride loss, 72 mM./L., and the difference becomes greater by 48 hours. Thiocyanate loss rates, exceeding inulin clearances, become greater as environmental chloride concentrations increase from 120 to 600 and 720 mM./L., indicating electrolyte loss by a pathway other than filtration. Blood concentration of SCN absorbed from 120 to 600 and 720 mM. Cl/L. fluids containing small amounts of NaSCN has not reached a plateau after 72 hours in 120, has become steady at 1.2 times environmental level in 360, and at 80% of environmental concentration in 600 and 720 in 36 hours.

Discussion

The absence of significant changes in the fresh weight of crabs exposed to environmental fluids of a wide range of electrolyte concentration makes it apparent that the volume of fluid within the animals is held constant although exchanges occur. The persistence of normal blood specific gravity in the ghost crab under such experimental conditions further indicates that there is no appreciable change in water or salt content of blood. Only after 24 hours in distilled water did this constancy of body weight and blood specific gravity show any signs of weakening. The significance of even these changes was questionable. The presence of regulation, therefore, is obvious.

Although there are statistically significant differences between the volumes of thiocyanate space, inulin space and total body water in the three species studied, there is no apparent correlation with dry or wet habitats. A lack of fundamental differences in the partitioning of water, SCN and inulin spaces and the implied cellular space, becomes apparent when these volumes are related to the volume of total body water.

The blood chloride concentration in the mangrove crab (*Goniopsis cruentatus*), significantly higher than those in the land crab (*Gecarcinus lateralis*) and the ghost crab (*Ocypode albicans*), bears a correlation to this crab's almost continuous exposure to sea water of high salinity. This correlation is also found by comparing mangrove crab and ghost crab urine chloride levels. It is interesting that urine taken from ghost crabs soon after capture on Nonesuch Island, Bermuda, did not differ appreciably in chloride content from that taken from the ones captured on the Delaware beaches. The difference in salinity of the sea water available to the two habitats does not impose a difference in urine chloride clearance. This might be expected in view of the brief nightly exposure to the surf during feeding. However, mangrove crabs, constantly exposed to 600 mM. Cl/L. sea water, did show the effect of high environmental salinity.

During the first two hours after injection, inulin became diluted in a volume of fluid about two-thirds the indicated thiocyanate space. This suggests that either (1) the blood SCN after injection is less concentrated, indicating a larger dilution volume, due to absorption of SCN by cells, or (2) inulin more slowly penetrates the remote spaces invaded more rapidly by SCN. The similarity of the slope of the dilution curves for massive and light SCN injections and the similarity between simultaneous SCN and inulin curves suggest that only the mechanical factors of spreading are involved. Recovery determinations on ghost crabs, accounting for 87 to 97% of injected SCN under a variety of environmental conditions, indicate that little, if any, SCN is bound by cells. Whether or not inulin eventually invades all of the SCN volume can only be suggested on the basis of data presented here. The apparent cessation of antennal gland activity in ghost crabs on sand appears to offer some opportunity for an answer. So far, it appears that in the 70 hours following the first two, inulin still occupies only two-thirds of the SCN space. The suggestion of a functionally closed circulation, inulin space, within the larger extracellular compartment, SCN space, is an interesting one for which the mechanical factors of lumen flow and stream boundary diffusion seem reasonable.

The breadth of the range within which two-thirds of the data are estimated to fall, presented in Table I, is taken to be a reliable indication of the effectiveness of regulation. Comparison of these ranges reveals that the three fluid compartments, total water, SCN space and inulin space, are more closely regulated in land crabs than in ghost crabs and mangrove crabs. A greater difference in the regulation of these volumes might be expected between ghost crabs and mangrove crabs in view of the difference in the stress imposed by their normal habitats. Chloride concentrations in the blood and urine of mangrove crabs are much more closely regulated than in land crabs and ghost crabs. This indicates that the land crab is farther along in the evolution of volume regulation and that the mangrove crab has a more definitive control of chloride concentration.

Comparison of chloride and SCN loss from the blood of crabs exposed to the various environmental fluids shows that these ions move at about the same rate in each species and in each situation. Urine SCN concentrations stood in the same ratio and range to blood SCN levels as did these respective concentrations of chloride. The graphs summarizing data are not further complicated by adding these items, inasmuch as they duplicate the chloride data. These observations indicate that it is valid to use SCN as "tagged chloride" in an effort to determine the movement of chloride ions under conditions of electrolyte and water stress. The presence of inulin or SCN in the blood did not affect the clearance, rate or degree, of the other. Inulin was not absorbed from the environmental fluids. Its presence in the environment did not affect the rate or degree of absorption of

SCN from the environmental fluids, or the rate or degree of inulin or SCN clearance from injected animals. This was true even when sufficient inulin was added to the environmental fluids to make them equal in concentration to the blood of animals injected for the determination of inulin clearance. Therefore, inulin was judged to exert no appreciable effect on the direction, rate or degree of electrolyte and water shifts in the concentrations used. The presence of SCN in the environment in concentrations used did not affect the rate or degree of inulin clearance, but it did affect the rate of fall of SCN levels in injected animals. In injected animals placed in fluids to which SCN had been added, blood concentrations of SCN fell more slowly and only to a point well above equilibrium with environmental SCN in 120 mM. Cl/L., about equal in 360 and well below in 600, but not cleared.

Of the three species, only the land crab and the ghost crab survived 24 hours out of water. This was to be expected from the differences in habitat and was one basis on which the three species were selected. The inulin and SCN clearance in land crabs and ghost crabs on sand for 72 hours, during which blood chlorides remained constant, indicate a difference in antennal gland activity. The indicated re-absorption of filtered water in land crabs could account for the lack of obtainable urine. If the re-absorption of electrolytes is obligatory, it could be a cause of the elevated chloride levels found in land crabs exposed to hypertonic environments. In ghost crabs, such a continuing filtration and re-absorption does not appear to exist. The dependence on contact with the sea for filtration and resulting urine formation is in agreement with the observations by Burger (1957) that haemoconcentration, from keeping lobsters in air, suppresses urine formation. His interpretation is that non-diffusible molecules in the blood draw in water principally through the gills, and that this water is bailed out as urine.

The similarity between inulin clearance rates in land crabs on sand and for 24 hours in distilled water is interesting. The same is true of ghost crabs. The persistent high inulin concentrations in these latter animals suggest very little filtration in distilled water. The possibility is immediately obvious that cellular osmotic swelling in gill membranes and branchial epithelium may cause mechanical, if not metabolic, interference with absorption of water by crabs in such environments. If this should be the case, why are mangrove crabs different?

Chloride and SCN loss in 0 mM. Cl/L. fluid, most rapid in land crabs and least so in mangrove crabs, appears to be compensated for by the absorption of available ion, SCN, from the environmental fluids. The rate and degree of net gain, blood concentration, of the absorbed ion is not proportional to the rate or degree of blood chloride, or SCN, loss. The three species clearly differ in their ability to retain normally present chloride ions and to absorb and hold SCN ions. The blood chloride level in land crabs seems to be least well held and the least well protected by absorption rates. The blood chloride of ghost crabs is somewhat better held and is better protected by a remarkably rapid absorption rate. Retention of blood chloride in mangrove crabs, best of the three, is supported by an intermediate absorption rate. The apparent superiority of the holding and compensatory mechanisms in mangrove crabs is reflected by their longer survival, past 48 hours. It should be pointed out, however, that in all three of these species, the net absorptions are inadequate to compensate for a falling blood chloride. The significance of some ion, however dilute, to the survival of crabs in 0 mM. Cl/L.

fluids is shown by the doubling of survival time by the retention in the blood of 7 mM. SCN/L. for 162 mM. Cl/L. lost in land crabs, 25 for 129 in ghost crabs and 9 for 72 in mangrove crabs during the first 24 hours of exposure. There was no such increase in the survival time of crabs in 720 mM. Cl/L. fluids to which similar amounts of Na SCN had been added. None of the animals showed any signs of depression.

Urine chloride of ghost crabs exposed to distilled water for 24 hours was 48% of blood concentration, indicating that some, though obviously not all, filtered chloride is re-absorbed. This is also indicated by comparable SCN data. The high urine chloride is not high enough to suggest chloride secretion by the antennal gland. The impression that these animals formed very little urine is supported by the fact that only 10% of the injected inulin is cleared during this 24-hour period. The less-than-blood concentration and the small volume of urine and the loss of one-third of blood chloride suggest a removal of chloride, and SCN, from the blood by a pathway other than the antennal gland. The urine chloride concentration in mangrove crabs, 62% of blood level, after a similar exposure indicates partial chloride recovery by the antennal gland. This re-absorption continues through 48 hours, but fails to repair a falling blood chloride concentration.

Comparison between a 35-gram ghost crab and a similar mangrove crab, calculated from data presented in Table I, serves to demonstrate the possible difference in pathways of chloride loss in animals exposed to distilled water. From Figures 1 and 2, it may be seen that 88 mgm. NaCl were lost from the blood of such a ghost crab during 24 hours in distilled water and that the concurrently formed urine contained 11 mgm. NaCl per cubic centimeter. For the chloride lost from the blood to have been cleared by only the antennal gland, 8.0 cc. of urine would have had to be formed. As calculated by inulin clearance, only 0.8 cc. of fluid was filtered during this period. Urine inulin concentrations were roughly equal to blood levels, indicating little or no water re-absorption or secretion after filtration. Ninety per cent of the chloride loss must have been by another route in the ghost crab. From Figures 1 and 2 it appears that 45 mgm. NaCl were lost from a comparable mangrove crab during a similar exposure and that the urine formed contained 15 mgm. NaCl per cubic centimeter. The filtration and excretion of 3.0 cc. of this urine would account for the blood chloride loss. According to inulin clearance, 3.5 cc. fluid were filtered, and according to urine inulin concentrations there was no appreciable re-absorption of water. In spite of the clearance of proportionately less chloride than water by the antennal gland, suggesting re-absorption of chloride, this is the only pathway necessary to account for the observed failure in chloride ion regulation in mangrove crabs exposed to distilled water. Re-absorption of chloride occurred in both species in distilled water. Since 0.8 cc. blood was filtered in the ghost crab, 18.0 mgm. NaCl crossed over into the lumen of the antennal gland. Since urine contained 48% blood chloride concentration, 8.6 mgm. NaCl were lost, and the remaining 9.4 mgm. must have been re-absorbed. The 3.5 cc. blood filtered in the mangrove crab carried 86.4 mgm. NaCl into the antennal gland. The urine, containing 62% blood chloride concentration, removed 53.6 mgm. NaCl, leaving 32.8 mgm. to be re-absorbed. This is in agreement with the observed chloride loss. The removal of more water than electrolyte from the blood of ghost crabs and mangrove crabs, and the decrease in blood chloride concentrations of all three species exposed to distilled water for 24 hours, make it apparent that the water entering the animals is flushing chloride out through the antennal gland. Moreover, loss of chloride through another pathway is indicated in land crabs and ghost crabs, but not necessarily in mangrove crabs.

Although complete extraction of electrolytes in one passage through the gill chamber can not be assumed, comparisons of absorption rates can be made. The absorption of SCN added to 0 mM. Cl/L. environmental fluids indicates a withdrawal from a volume of environmental fluid equal to the SCN space, about 11 cc. for a 35-gram animal, in 2 hours for ghost crabs, 6 hours for mangrove crabs and 15 hours for land crabs. Leveling off of the concentration curves in time suggests that if absorption rates hold, the rate of diffusion outward increases with increasing concentration. This suggests a far more rapid turnover at the gill membrane and, perhaps, branchial epithelium than clearance rates in the antennal gland would indicate. The gill and, perhaps, branchial epithelium appear to be the site of this absorption activity since animals whose digestive tracts were closed at both ends with grafting wax did not differ in absorption rates from those animals not blocked. Similar blocking prior to electrolyte and inulin loss determinations indicated that the digestive tract has no significant role in the clearances observed.

The appreciable, 10% rise in blood chloride concentrations in land crabs exposed to 120, 240, and 360 mM. Cl/L. fluids indicates that the rate of absorption of chloride from these environments exceeds the rate of loss until a new steady-state is reached. The steadily maintained higher blood level, failing only in 120 mM. Cl/L. at 72 hours, shows that the regulation is effective, if not compensating. The much more elevated, 20% higher, yet steady concentrations found in animals exposed to 480 mM. Cl/L. for 72 hours, 600 for 48 and 720 for 24, indicate that this regulation persists and has some flexibility and upper limits in situations hypertonic to the blood. Absorption rates are greater than indicated by the concentration curves, for it must be assumed that during absorption the ions are being lost at rates suggested by the SCN loss curves. The slower rate of SCN loss from injected animals, the slower rate of SCN absorption by uninjected ones and the slower rate of filtration in 600 and 720 mM. Cl/L. fluids indicate that there is reduced exchange with the environmental fluids perhaps due to reduced exposure which in turn may be due to a partial restriction of gill chamber volume or flow as suggested by the work of Gross (1957) on the brachyuran shore crab (*Pachygrapsus crassipes*) exposed to hypertonic fluids. The loss of ions across the gill membrane and, possibly, the branchial epithelium and the persisting, though reduced, filtration through the antennal gland are not sufficiently rapid to prevent an accumulation of ions from the environment resulting in the elevated blood chloride level observed. Although the lack of urine data precludes further analysis and appraisal of this regulation, it appears that there is a correlation between the dry habitat of land crabs and their relatively slow electrolyte clearance resulting in elevated blood chloride levels even in hypotonic environmental fluids.

The regulation of blood chloride concentration in ghost crabs is more rigid from 120 to 600 mM. Cl/L. than in land crabs. The similarity of the SCN loss curves for 360 to 720 mM. Cl/L. fluids, faster than inulin clearance, indicates that the antennal gland is of only secondary importance in electrolyte loss in near-isotonic and hypertonic environments. Since in all fluids the injected SCN is cleared in about 24 hours, the constancy of the blood chloride concentration would appear to depend on the net absorption, or retention of the same quantity of electrolyte irrespective of environmental concentration. This can be concluded to happen from the net absorption curves. Proportional to chloride ions present in environmental fluids, there is about three times as much SCN in 120 mM. Cl/L. fluids as in 360 and almost twice as much in 360 as in 600. This is approximately the ratio of net absorption concentration of SCN accumulating in the blood during exposure to the various environments. The greater volume of environmental water involved in this extraction process in 120 mM. Cl/L. fluids is reflected in the more rapid filtration through the antennal gland.

Blood chloride is held constant over a wider range, 120 to 720 mM. Cl/L., for 72 hours in mangrove crabs than in either of the other two species. The close approximation of urine chloride concentrations to those of environmental fluids suggests that the regulation is closely held and yet flexible in that absorbed ions are apparently retained in hypotonic situations and cleared in hypertonic ones. The fact that filtration continues at the same rate for all environmental fluids, even distilled water, shows that constant blood chloride levels must be maintained by prompt re-absorption of ions and water by the antennal gland and by absorption and loss by any other route of exchange involved. The more rapid loss of SCN in hypertonic environments than in near-isotonic ones, and these more rapid than in hypotonic ones, at a time when blood chlorides are constant, shows that the turnover, absorption and loss, of electrolytes is more rapid in the more concentrated environments. This may account for the fact that the accumulated SCN curves fail to level off at points which suggest the ratios of proportionate SCN and chloride concentrations, as was found in ghost crabs. The outbound passage of the same amount of water through the antennal gland in all environmental concentrations, indicated by inulin clearance, fails to account for electrolyte clearance, except from crabs in distilled water.

The fact that urine chloride concentrations approach environmental fluid levels and not blood levels during exposure to 120 to 720 mM. Cl/L. environments for up to 48 hours in ghost crabs and 72 hours in mangrove crabs suggests that the antennal gland re-absorbs some chloride in hypotonic and some water in hypertonic situations after filtration. It is apparent that the reabsorption of chloride is not completely adequate in hypotonic environments in either species and begins to fail earlier in ghost crabs than in mangrove crabs. The re-absorption of water in hypertonic environments is more effective in both species. Urine electrolyte and inulin concentrations indicate that the high urine chloride in hypertonic and near-isotonic environments is due to re-absorption of water. Inasmuch as blood chloride levels continue to be maintained, and inulin data indicate only a moderate increase in filtration and only a moderate decrease in water re-absorption by the antennal gland, the markedly reduced level of urine chloride in hypertonic environments at 72 hours implies a closing of a portal of entry of chloride in the ghost crab. This might be due in part to restricted gill chamber exposure suggested in the shore crab in hypertonic fluids by Gross (1957). The marked increase in urine chloride concentration in the ghost crab in 120 mM. Cl/L. fluid in 72 hours, when blood chloride level remains constant and urine inulin concentrations indicate no re-absorption of water, suggests accelerated chloride absorption from the environment. It is interesting that urine chloride concentrations in the two species are near normal when the mangrove crab is in 600 mM. Cl/L. fluid, its normal habitat, and when the ghost crab is in 360 mM. Cl/L. fluid, near isotonicity with its blood. It is also interesting that urine and blood chloride concentrations are equal when the animals are exposed to environmental chlorides 100 mM. Cl/L. less concentrated than the blood. This gives a rough estimate of the re-absorption gradient in the antennal gland and indicates that similar mechanisms and thresholds are involved in the two species.

When animals of these three species are exposed to environmental fluids ranging from 120 to 600 mM. Cl/L., the rate of turnover, absorption and loss, of electrolytes and the rate of filtration are less in the land crabs than in the others. The difficulty in getting urine samples suggests re-absorption of most of the filtered water, which might be expected in view of this crab's adaptation to a dry habitat. The electrolyte turnover and filtration rates are most rapid in the ghost crab in hypotonic and in the mangrove crab in hypertonic environmental fluids. There is an apparent correlation between the almost constant exposure of the mangrove crab to sea water hypertonic to its own blood and a rapid turnover and clearance rate. It appears that the defense in the ghost crab is against the inbound movement of hypotonic fluids and that this is a poor defense at best in view of the inefficient re-absorption of chloride by its antennal gland. It is interesting that when animals of these species are exposed to environmental fluids which are near isotonic to their own blood concentrations, the filtration rates through the antennal glands are similar. This indicates that the hydrostatic and osmotic factors in filtration are similar in all three of the species. This augments the interpretation based on the uniformity of re-absorption gradients that similar mechanisms and thresholds are involved in antennal gland function in the three species.

In the early intervals of SCN absorption and loss determinations, before blood levels are much altered, absorption rates exceed loss rates in ghost crabs and mangrove crabs and are about equal in land crabs in hypotonic, 120 mM. Cl/L., fluids. Land crabs and ghost crabs hold about equal in near-isotonic, 360 mM. Cl/L., fluids, but mangrove crabs show an absorption advantage in the same fluid, which is hypotonic to their blood. Early loss rates exceed early absorption rates in all three species in hypertonic, 600 mM. Cl/L., sea water. These absorption and loss rate differences are parallel to the leveling-off points in the SCN accumulation curves, which are interpreted to arise from the equating of outbound and inbound passage of ions across gills and, perhaps, branchial epithelium as blood concentrations are increased as a result of absorption exceeding loss earlier. Comparison of these leveling-off concentrations of net absorbed, accumulated, SCN and the ratio of the concentration of chloride maintained in the blood and that imposed by the environmental fluid shows close agreement for near-isotonic and for hypertonic situations. In both the ghost crab and the mangrove crab, the plateau has not been reached in hypotonic fluid, but in the land crab there is evidence of both a leveling-off and a breakdown in blood chloride regulation at 72 hours. In crabs of all three species exposed to distilled water, the plateau is so remote and the breakdown so severe that no conclusions can be drawn. Leveling-off of loss curves in time is interpreted to reflect rates markedly reduced by the falling blood concentration. After ten days no SCN or inulin could be found in injected animals kept in the laboratory under normal conditions. The evidence from the net absorption curves is that electrolyte movement is rapid and precise. In hypotonic environments an appreciably longer time is required to reach a plateau than is required to clear the ion once it is injected, which suggests that there is a choke on the rate of absorption of ions from hypotonic fluids. This is altogether reasonable when the handling of the required amount of fluid is considered.

The loss of injected SCN to a level well above a concentration in equilibrium with SCN added to 120 mM. Cl/L. environmental fluid coincides with and supports the evidence from urine chlorides of animals in such environments that electrolyte loss continues even in situations where ions must be acquired to maintain constancy. The fact that injected SCN falls to approximate equilibrium with environmental SCN in animals exposed to near-isotonic fluids also supports this evidence. The loss of injected SCN to a concentration less than environmental in animals in 600 mM. Cl/L. fluids, in which absorbed SCN is held to less than equilibrium concentration, shows that the capacity to lose electrolyte is not saturated by this degree of hypertonicity. It is clear that these loss rates are much greater than can be accounted for by passage through the antennal gland.

On the basis of early clearance rates, before blood concentrations are greatly decreased, fluid equal to the inulin space volume is filtered by the antennal gland of land crabs in their normal habitat on sand in about 60 hours, and in mangrove crabs in 600 mM. Cl/L. sea water, their normal habitat, in 24 hours. In ghost crabs on sand no appreciable filtration was found. However, in sea water between 360 and 600 mM. Cl/L., to which ghost crabs normally have access, the filtration rates are similar to those of mangrove crabs. This indicates the importance of the ghost crab's brief nightly exposure to the surf. It is assumed that in their normal habitat, ghost crabs filter somewhat slower than do mangrove crabs, but that they do filter is apparent from the fact that fresh-caught crabs have urine. Therefore, an obvious correlation exists between filtration rate and type of habitat in these three species.

The turnover rates indicate the activity in electrolyte and water movement which goes on during the maintenance of constancy of volumes and concentrations in the water compartments measured. The persistence of normal values for these quantities in the variety of devised and imposed environmental stress situations is as remarkable as the rate of continuous change which underlies it. It must be concluded that in submerged crabs of these three species, the gills and, possibly, the branchial epithelium provide the principal pathway for this rapid and precise absorption and loss of electrolytes and water, and that the antennal gland plays only a limited role in this turnover. However, the urine chloride, thiocyanate and inulin concentrations indicate that clearance through the antennal gland may provide the all-important fine adjustment in blood concentration of electrolytes and water.

Among these three species, found in different degrees of exposure to seas of different salinity, the mangrove crab, most constantly and continuously exposed intimately to the stable environment of the sea, is the one showing the greatest capacity to regulate the concentration of blood chloride when subjected to environments of widely differing salinities. The crab most independent of the sea, the land crab, has the most definitively regulated volume of total, inulin space and SCN space water, and an adequate electrolyte regulation when exposed to a limited hypotonic range or to food containing proportionately more water than salt, but little or none when environmental chloride exceeds that of blood as does the sea water accessible in its habitat. In the ghost crab, intermediate between them, there appear to be the mechanisms for effective regulation with diminished chloride absorption in hypertonic fluids and increased absorption in hypotonic ones, but with the threat of an extravagantly wasteful chloride loss through the antennal gland. The independence of the land crab from the sea depends on the maintenance of a gradient and not on effective regulation. The land habitat of the ghost crab is critically dependent on access to the surf, albeit for a short nightly exposure. The sea habitat of the mangrove crab is a complete commitment despite a wide range of effective regulation. There emerges a picture of independence which depends on the constancy of a normal gradient, and the capacity to tolerate a changing gradient which depends on effective regulation. They afford the mechanisms of adaptation to totally different habitats.

SUMMARY

1. Exposure to environmental salinities ranging from 120 to 720 mM. Cl/L. for 72 hours did not produce changes in fresh weights of the land crab (*Gecarcinus lateralis*), the ghost crab (*Ocypode albicans*) or the mangrove crab (*Goniopsis cruentatus*). There was an increase in weight of questionable significance after 24 hours in crabs exposed to distilled water. Only in distilled water was there any change in the blood specific gravity of ghost crabs. Even this change was of questionable significance.

2. The total body water content of ghost crabs is significantly larger than those of land crabs and mangrove crabs, which are similar. The fractions of total water content which are available for the dilution of thiocyanate and inulin are similar in the three species. The volumes available for the dilution of inulin are about two-third the volumes in which SCN appears to be diluted. This suggests the interesting possibility of a functionally closed, lumen flow, circulation.

3. The blood chloride concentration of mangrove crabs, although less than that of their environment, is significantly greater than those of the more terrestrial ghost crabs and land crabs, which are similar. The urine chloride concentration of mangrove crabs is identical to that of its environment and is more concentrated than that of ghost crabs.

4. Exposed to environmental fluids of 120 to 600 mM. Cl/L. sea water for 72 hours, land crabs show adequate regulation of blood chloride concentration over a limited hypotonic range, but little or no regulation in fluids hypertonic to its blood chloride. Blood chloride regulation in ghost crabs is adequate over this range, but with the production of a urine which wastes chloride in hypotonic fluids. Mangrove crabs show an adequate and closely held regulation of blood chloride concentration in this range and the production of a urine with chloride levels similar to those of the environment, but with some chloride leakage in hypotonic fluids. Blood chloride regulation failed in all three species when exposed to distilled water for 24 hours, and in land crabs and ghost crabs exposed to 720 mM. Cl/L. for about 24 hours. Mangrove crabs survived 72 hours in 720 mM. Cl/L. fluid with regulation intact, but could not survive 24 hours in air.

5. On dry sand, land crabs filter across the antennal gland a volume equal to their inulin space in 60 hours. It also re-absorbs most of the water of the urine thus formed. This is not true of ghost crabs in which the formation of urine appears to depend on water gained during brief nightly exposures to the surf. When

exposed to 600 mM. Cl/L. sea water, their normal habitat, mangrove crabs filter their inulin volume in 24 hours. There is an apparent correlation between these filtration rates and the availability of water in the habitat.

6. Antennal gland filtration and re-absorption rates are adequate to account for the rate of chloride loss in mangrove crabs in distilled water. This is not true for ghost crabs and land crabs in which filtration rates are not much faster than those on sand. Electrolytes are escaping across some other membrane, supposedly gills and, perhaps, branchial epithelium. The loss of electrolyte by a route other than the antennal gland is also apparent in animals of all three species exposed to environmental fluids from 120 to 720 mM. Cl/L.

7. Re-absorption of chloride by the antennal gland of ghost crabs and mangrove crabs exposed to hypotonic fluids and of water in animals exposed to hypertonic fluids is apparent from the similarity between urine and environmental chloride concentrations. Similar re-absorptions can be inferred from data presented on land crabs.

8. The similarity of the mechanisms and thresholds involved in antennal gland function is indicated by (1) the approach of urine chloride concentrations to the blood chloride levels when ghost crabs and mangrove crabs are exposed to environmental fluid chloride levels 100 mM. Cl/L. less concentrated than the blood, and (2) the similarity in filtration rates in all three species when animals are exposed to environmental fluids which are near isotonic to their own blood chloride concentrations.

9. The blood concentrations of SCN absorbed from 120 to 720 mM. Cl/L. environmental fluids tend to plateau, due to equating of inbound and outbound ion passage, at a point roughly equal to the ratio between blood chloride and environmental chloride levels. The point of plateau is reached more slowly in hypotonic situations indicating the difficulty of handling the required volume of environmental fluid. The persistence of electrolyte loss, even in situations where ions must be rapidly absorbed to maintain constancy, is indicated by the SCN loss rate curves for the various environments.

10. The rates of turnover of water and electrolyte are as remarkable as the constancy of the regulation from which they result and for which they are responsible. The effectiveness of this regulation in mangrove crabs and the maintenance of a concentration gradient in land crabs can be related to the successful adaptation of these two species to totally different habitats.

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REGIONAL LOCALIZATION OF NEURAL AND LENS ANTIGENS IN THE FROG EMBRYO IN RELATION TO INDUCTION

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A number of embryologists recently have attempted to characterize embryonic cells by their constituent proteins. This approach is of particular interest when this characterization is attempted before, or at the time of, embryonic determination since it might be expected that a protein, or proteins, usually associated with a given tissue would increase in amount once the differentiation and growth of that tissue has already begun. The serological experiments of Ebert *et al.* (1955) in localizing cardiac myosin and actin in the early chick blastoderm, and those of Ten Cate and Van Doorenmaalen (1950) in determining the time of appearance and location of the lens antigen in frog and chick embryos are examples of this approach.

In relation to embryonic induction it would appear to be of some theoretical significance to be able to map or localize the protein that may characterize the reacting tissue in an induction system. In the induction of the medullary plate by the underlying chorda mesoderm, where is the greater amount of neural antigen localized just before this induction occurs? Is there more specific neural protein in the inductor (chorda mesoderm) or in the reacting tissue (gastrula ectoderm)? If the inductor has more neural protein, then this may imply the passage of specific protein from the inducing to the reacting tissue and subsequent synthesis of this protein in the reacting tissue. If, on the other hand, more of the neural antigen is present in the gastrula ectoderm, this implies that the induction stimulus is of a less specific nature and may merely be an activator for the synthesis of more neural protein in the reacting tissue.

MATERIALS AND METHODS

In order to examine this question, antisera were developed against adult male frog brains (*Rana pipiens*) and antisera developed against adult frog lenses and cattle lenses for a previous investigation (Flickinger *et al.*, 1955) were also utilized. The method of preparing the anti-lens sera was presented in the above paper. Two antisera against adult male frog brains were prepared by cutting open the brains and freeing them of all visible blood, washing them several times in cold 0.65% saline, homogenizing them in a glass tissue grinder in the cold, and injection of the supernate obtained from centrifugation at 3000 g. This supernate was about 1% protein as shown by nitrogen determinations. Intravenous injections of 0.5, 1.0 and 2.0 ml. and an intraperitoneal injection of 4.0 ml. were given on alternate days and constituted an injection series. Three such series of injections were administered a week apart with the modification that the whole uncentrifuged homogenate was injected intraperitoneally in the third series of injections. The rabbits were bled 7 days after completion of injections. One of the antisera reacted with a 0.1% protein supernate from adults' brains at an antiserum dilution of 1/128. Obviously extracts of adult frog brains are not highly antigenic.

In the preparation of test antigens, early gastrulae (Shumway stage 10) were operated upon under sterile conditions and cut in four regions; ectoderm, dorsal mesoderm, ventral mesoderm and endoderm, as seen in Figure 1. Most of the large white yolky cells were removed from the ventral and dorsal mesoderm tissues. Recently hatched larvae (Shumway stage 19–20) were cut into three parts; head, trunk and gut (Fig. 1). The heads were removed by cutting vertically just posterior to the gill plate; the trunks were separated from the guts by cutting horizontally just ventral to the somites. No attempt was made to count the number of gastrulae and larvae that were operated upon, but for each preparation of test antigen from an embryo fraction, several hundred of the appropriate tissues were homogenized with an equal volume of 0.9% NaCl in micro-tissue grinders and then centrifuged in micro-centrifuge tubes (3 mm. inside diameter × 55 mm. length) in adaptors designed for the 7-ml. high speed head of the International centrifuge. After removal of the pigment, yolk, and lipid cap by repeated centrifugation at 15,000 g, some 0.2–0.3 ml. of a centrifugal supernate is obtained which can be used as the test antigen. The precipitin reactions were carried out in the same type of small tubes used for the centrifugations so as to conserve the antigen preparations. In some cases more concentrated embryo antigen preparations were obtained by homogenizing the embryo parts with an equal volume of supernate from a previous fractionation of the same tissues. A number of nitrogen determinations were made upon the test antigen preparations by the nesslerization method. The protein concentrations ranged from .35–1.5% protein, but for any given fractionation the protein concentrations were usually fairly similar for the four parts of the gastrula or the three regions of the larva. The test antigen preparations were adjusted to the same protein concentration before any given set of serological tests.

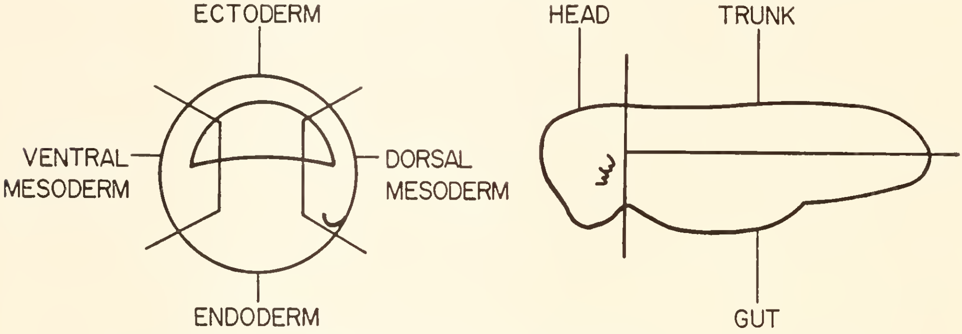

FIGURE 1. See text for explanation.

RESULTS

The anti-brain serum was not organ-specific and cross-reacted with frog serum, muscle, heart and kidney. It was found that serial absorption of 1.0 ml. of anti-brain serum with a total of 1.15 ml. of female frog serum, 1.0 ml. of a centrifugal supernate from a frog heart homogenate and 0.95 ml. of a frog kidney supernate was sufficient to render the anti-brain serum specific. If the frog serum was not used in the absorptions then it required 2.4 ml. each of the heart supernate and the kidney supernate to render 1.0 ml. of the anti-brain serum organ specific. After absorption the antiserum did not react with centrifugal supernates obtained from adult frog heart, kidney, muscle, liver, spleen, ovary and frog serum.

In four separate experiments where the anti-adult male frog brain serum had been absorbed to completion with male frog serum, the antiserum showed a precipitin reaction with female frog serum. This was also found to hold true for similar absorption of an anti-adult male muscle serum. Upon further dilution with male frog serum the reactivity towards female frog serum disappeared. If the anti-brain serum was diluted 1/4 with normal rabbit serum and reacted against male and female frog sera, the latter reaction occurred at once while a faint precipitate did not appear in the male serum reaction for a period of twenty minutes. At the end of an hour the female serum-antiserum precipitate was much heavier than the male serum-antiserum reaction. It is known from agar-plate serology experiments of Flickinger and Rounds (1956) that an anti-embryonic yolk fraction serum gives 5 precipitate bands with female frog serum, and only two with male frog serum, so that the female frog serum apparently contains some proteins in higher concentration, or with different determinate groups, than those found in serum of the male frog. It is more difficult to understand the situation in this work where the antibodies have been formed in response to injections of an adult organ from a male frog. It might be expected that absorption to completion with male frog serum would not only remove activity to the male serum but also to female serum, especially since the serum contaminating the injection antigen was male serum. The most likely explanation is that the common frog serum proteins in male and female are in higher concentration in the female serum and therefore the female serum is a better antigen and can still react with anti-adult male organ sera absorbed to completion with male frog serum. This explanation seems more plausible than trying to invoke any type of pangenesis for adult organ antigens.

Anti-brain serum, rendered specific by absorption, reacted positively with all test antigen preparations from the four parts of the gastrula and the three regions of the larva (Fig. 1). The precipitates in every case were distinct after twenty minutes at room temperature; after twenty minutes at 37° C. and after twenty minutes at 6° C. the precipitates were still of equal intensity. Normal rabbit serum-antigen controls were negative. No attempt was made to titer the antiserum against the various test antigen fractions since it was apparent that the antigens reacting with the brain-specific antiserum were to be found in all regions of the gastrula and larva in approximately equal amounts. Absorption of previously unabsorbed anti-brain serum with the antigen fraction from the larval gut (where no elements of the nervous system are present) also removed activity against all other antigen fractions, thus indicating in another way that proteins bearing the determinate groups that react with the anti-adult brain serum are located in all regions of the frog gastrula and larva.

In order to see if different proteins may be given off by different tissues, as some of the work of Niu (1956) suggests, a series of forty explantation experiments was carried out in which 20–30 pieces of larval brain (stage 19) or larval trunk (Fig. 1) were stripped of their epidermis and cultured in the bottoms of deep well depression slides in Niu-Twitty solution (1953). After a week of explantation the culture fluids were collected from above the tissues, centrifuged, and the supernates from the various cultures of denuded heads were pooled, as were the trunk culture supernates. There was some slight cytolysis in these cultures which would account for the release of some soluble protein into the medium, but it is felt that some of the protein probably was released into the medium by "natural means" other than cytolysis. These solutions were dialyzed against distilled water and then evaporated in dialysis bags suspended in front of a fan in a cold room (2–4° C.). An individual dialysis bag usually contained about 8 ml. of the culture supernate and this volume was reduced to about 0.2 ml. Nitrogen determinations in one case revealed the protein level of the concentrated supernate to be 0.06% protein. The absorbed brain-specific antiserum gave positive ring tests of equal intensity with both head and trunk supernates twenty minutes after the test antigens were layered over the antiserum. It would appear that the proteins given off from the denuded heads and trunks bear similar determinate groups that react with the brain-specific antibodies. These results are certainly not definite enough to state that proteins or nucleoproteins (inducing substances?) given off by different inductors are similar, even though much of the protein from the trunk cultures would be derived from the exposed myotomes and that from the head cultures would come from the brain, since the antiserum lacks the desired degree of specificity. However, the data tend to support the many biological examples of non-specificity of the inducing agent (Holtfreter, 1951).

The obvious disadvantage to the use of anti-brain serum is its lack of specificity. Hence it was decided to use anti-lens sera where it is known that this organ has a greater degree of organ specificity. This organ is of course also an induced organ and the localization of lens antigen in the embryo would be of value in relation to the induction problem. In a previous investigation (Flickinger *et al.*, 1955) anti-frog lens serum was used to demonstrate the presence of lens antigen in the anterior half, but not in the posterior half, of feeding frog larvae. However, the negative results do not necessarily mean an absence of the lens antigen but might imply only a reduced amount of the antigen in the posterior region of the embryo.

As a first attempt to improve the means of localizing lens antigen, flank ectoderm, which is known to possess the ability to respond to an induction stimulus and form lens, was stripped from several hundred hatched larvae (Shumway stage 19), homogenized in 0.9% NaCl, and a supernate fraction obtained by centrifugation. This extract did not react in the ring tests with an anti-cattle lens serum, or an antiserum to cattle lens α- crystalline (previously provided to me by Dr. Ten Cate of the University of Amsterdam). The anti-α-crystalline was also negative against the gastrula ectoderm and chorda mesoderm test antigens. It is believed that the epidermis alone did not provide sufficient soluble protein to give a precipitin reaction. Therefore, in order to concentrate the test antigen preparations, it was decided to use test antigens from the head and trunk regions of hatched larvae in which the tissues were homogenized with an equal volume of supernate of heads or trunks from a previous fractionation. This has an advantage over the use of posterior halves of larvae (Flickinger *et al.*, 1955) in that the less metabolically active gut region, containing more storage protein, is not included with the trunk tissues.

The anti-frog lens serum gave immediate strong ring tests with both the head and trunk antigen preparations, but this antiserum also reacted with adult frog serum and therefore was not organ-specific. After absorption of 1 volume of anti-frog lens serum with ½ volume of female frog serum the antiserum was negative to serum, and still gave definite ring tests after 20 minutes with head and trunk supernates. This antiserum was negative against test antigen preparations from adult muscle, heart, kidney and ovarian supernate, but it did give a weak positive reaction with an adult brain supernate after one hour. Therefore three volumes of the anti-frog lens serum were absorbed with two volumes of a centrifugal supernate obtained from homogenizing adult frog brains with an equal volume of frog serum. This absorption left the anti-frog lens serum negative to serum and brain and the antiserum gave a reaction with the test antigen preparation from larval trunks.

The use of antiserum against adult frog lens in localizing the presence of lens protein, or a protein bearing lens determinate groups, has the disadvantage that the antibodies are directed against determinate groups characteristic of frog proteins as well as those characteristic of lens protein. It would be preferable to use an organ-specific anti-cattle lens serum where, if the antibodies did react with frog embryo test antigens, the reaction would most likely be due to the lens determinate groups of the frog antigens reacting with the antibodies directed against cattle lens proteins.

The anti-cattle lens serum was tested against frog serum, brain and kidney and found to give no reaction. However, this antiserum gave immediate strong positive reactions with the concentrated head and trunk antigens from the hatched larva. The appropriate normal rabbit serum-test antigen controls were negative. The anti-cattle lens serum did not react with test antigen preparations from the four parts of the early gastrula. This negative result might be explained by the fact the gastrula supernates were not prepared as concentrated antigens, as in the case of the larval heads and trunks, but it is also likely that the lens antigen is present in lower concentration at earlier stages.

In looking back at the previous work with this antiserum (Flickinger *et al.*, 1955) it was noted that the antiserum reacted with supernates from both the anterior and posterior halves of 69-hour chick embryos as well as with the ovarian supernate and hatching larva supernate from frog embryos.

DISCUSSION

If antigens with neural determinate groups are localized in all parts of the gastrula and tailbud larva, and particularly in the more critical case where antigens with lens determinate groups are situated in the trunk region (somites, neural tube, notochord, and dorsal epidermis) of the early larva, this indicates that organ antigens may be more disperse for a certain time than the organ-forming areas in the embryos. Ebert *et al.*, (1955) have shown this to be the case for cardiac myosin in the chick blastoderm, although cardiac myosin did become localized in the heart-forming areas after a period of time. Cardiac actin was always confined to these heart-forming regions.

It seems that the presence of organ antigens outside their organ-forming districts does not invalidate the idea that one may in part characterize a cell, tissue or organ by the structural and functional (enzymes) proteins they contain. The wide distribution of organ-specific proteins is an indication of the totipotency of various regions of the embryo which has been demonstrated by numerous transplantation experiments. It would also tend to support the idea that embryonic induction could be any one of a number of stimuli which might evoke protein synthesis at a particular region in the embryo.

For example: any specific protein, which the genetic machinery of the cells would allow to be synthesized, might be stimulated (induced) to this synthesis by a number of factors. Ribonucleic acid or ribonucleo-protein is a critical component for protein synthesis (Brachet, 1950; Gale and Folkes, 1954) and for embryonic induction (Niu and Twitty, 1953). It is known that protein synthesis is an endergonic process demanding energy (Fruton and Simmonds, 1953) and Miller (1939) has demonstrated the reversal of the anterior-posterior polarity of a section of stem of a regenerating hydroid by raising the temperature (and therefore the level of energy-yielding reactions) at the posterior end of the stem. The presence of free amino acids is known to be necessary for adaptive enzyme formation (Halvorson and Spiegelman, 1953) and it is known from the work of Barth (1941), Holtfreter (1945), Yamada (1950) and Flickinger (1958) that competent tissues can be stimulated to differentiate independently of an induction stimulus from another tissue by pH shock treatments which can dissolve yolk (Holtfreter, 1946). Flickinger (1957) has emphasized that the solubilization of yolk protein, which provides the material from which cytoplasmic proteins are synthesized, can be a causal step in embryonic induction. Even after specific cytoplasmic proteins have been synthesized the provision of an enzymatic substrate, as in Wilde's (1955) conversion of gastrula ectoderm cells into melanophores by giving them phenylalanine, could be considered an embryonic induction. Activators or inhibitors of an enzymatic reaction might then also act in an induction system. Viewed in this manner there may be multiple aspects of embryonic induction with any factor, or combination of factors, that would facilitate the synthesis, or activity, of specific proteins being considered an inductor. This is somewhat similar to the case shown by Spiegelman and Reiner (1947) where, under conditions optimal for growth and protein synthesis, adaptive enzymes may be formed without the substrate or inducer being present.

If a sub-differentiation threshold level of any given specific protein, or proteins, exists throughout the embryo, it may be that a preferential hierarchy of cell and tissue specialization exists such that when conditions become optimal for protein synthesis in a given region of the embryo then a specific cell type or tissue will be formed. That there is some kind of preferential hierarchy can be seen from the tendency for gastrula ectoderm activated by sub-lethal cytolysis to form forebrain structures (Holtfreter, 1944). This tendency to form head structures first can be seen in Sabella regeneration (Berrill, 1931) where the most anterior part forms first and then fills in the missing parts. From studies of regeneration and embryonic development it is evident that differentiation occurs time-wise along anterior-posterior and dorsal-ventral axes with the anterior and dorsal differentiations usually preceding the posterior and ventral ones. Possibly these anterior-posterior and dorsal-ventral patterns of specialization are due to gradients of factors which favor protein synthesis, and that certain types of cell or tissue specialization are favored when conditions for protein synthesis become optimal. For example, Flickinger (1957) has hypothesized that the primary organizer area forms where the first and most active conversion of yolk to cytoplasm occurs. The biological totipotency of most parts of the embryo, and the serological evidence tend to indicate that some organ-specific proteins may be more widely distributed than the corresponding specific organ-forming areas. Possibly the postulated sequential protein synthesis, and cell specializations and growth that may depend upon these syntheses, are of a self-limiting type as postulated by Rose (1952, 1957) and Weiss (1952). The question why the synthesis of a given type of protein may be favored when conditions become optimal for protein synthesis is indeed puzzling. It is apparently not due to a purely qualitative distribution of protein, or to unequal nuclei (Briggs and King, 1952). It might be ascribed to a quantitative distribution of various organ-specific proteins, or nucleoproteins, but although there are gradient-wise distributions of soluble proteins and ribonucleoproteins, there is as yet no evidence concerning the specificity of these compounds. Another possibility might be a preferential sequence of activity of different specific genes.

It may be well at this time to review the idea of Driesch that the fate of a cell or tissue is a function of its position. It is well known that undetermined embryonic cells and tissues tend to "fit in" to the particular locale in which they find themselves. The fact that the nuclei of the cells of the determined neural plate are apparently undifferentiated and still able to promote complete development when injected into the enucleated egg (King and Briggs, 1954) and the apparent determination of the whole mouse embryonic shield before the determination of its individual constituent cells (Grobstein, 1952) argues for some sort of "supracellular patterning," perhaps of a polar or axial type (Child, 1941; Rose, 1957), which precedes cell specialization. This is a question which deserves a good deal of attention from embryologists.

SUMMARY

1. Anti-adult male frog brain and muscle sera absorbed to completion with male frog serum still react with female frog serum. It is believed that serum proteins common to the male and female may be in higher concentration in the female serum and hence account for this reaction.

2. Anti-brain serum, rendered organ-specific by absorption, reacted positively with test antigen preparations from four regions of the early frog gastrula (ectoderm, dorsal mesoderm, ventral mesoderm, and endoderm) and three regions of the hatched frog larva (head, trunk and gut). The proteins bearing brain determinate groups are apparently situated throughout the embryo at these stages.

3. The organ-specific anti-brain serum gave positive precipitin reactions with culture supernates from both larval heads and trunks which had been denuded of their epidermis and explanted for a period of a week. The proteins given off by these cultured heads and trunks bear similar determinate groups that react with the brain antibodies.

4. An absorbed organ-specific anti-frog lens serum, and an organ-specific anti-cattle lens serum, reacted with concentrated test antigen preparations from both the heads and trunks of hatched frog larvae. It seems that lens antigen, or protein bearing lens determinate groups, is localized in areas other than the lens-forming region.

5. The significance of these results is discussed.

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STUDIES ON NEUROMUSCULAR TRANSMISSION IN LIMULUS

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Among invertebrates only certain crustaceans and insects have been the subjects of detailed study in regard to neuromuscular mechanisms. There have proved to be very considerable differences between the various arthropod mechanisms encountered on the one hand (Wiersma, 1957—Crustacea; Hoyle, 1957—insects) and those of vertebrates on the other (Fatt, 1954). The differences contribute to the difficulty in arriving at a general concept of the way in which coupling between excitation of the surface membrane of the muscle fiber, which is achieved by nervous action, and shortening of the contractile material, is brought about. But they also show that certain favored hypotheses in regard to vertebrate muscle are either of only limited applicability for muscle as a whole, or are wide of the mark. There is a strong difference of opinion regarding the relevance of the electrical activity of the muscle fiber membrane in the process. Most recent authors (*cf.* Sten-Knudsen, 1954; Huxley, 1956) have regarded the contractile machinery as being in some way connected with the membrane potential. Some (*e.g.*, Csapo and Suzuki, 1957) believe that contraction is initiated by current flow resulting from membrane action potentials. For the Crustacea, it has been found necessary to postulate a separate coupling mechanism within the muscle fiber which may be activated differently by neuromuscular transmitter action in different cases. In some (Hoyle and Wiersma, 1958b) there may be a direct action by the transmitter substance on the coupling mechanism, the electrical intermediate (or propagation) stage having been by-passed. In others, electrical changes, or the ionic fluxes associated with them, affect the coupling mechanism.

From this it seems likely that in the elucidation of the general problems of excitation-contraction coupling, the arthropods will provide favorable material. In them single muscle fibers are innervated by more than one motor axon, each having different motor effects, and in the Crustacea there are also inhibitory axons which uncouple the excitatory action. In many arthropod systems the unit of contraction is not an all-or-nothing twitch, and contractions are minutely graded. This difference between arthropod muscle and ordinary skeletal muscle of vertebrates is probably attributable to the absence of propagated muscle action potentials in the former. In spite of their potential interest, and the variety of their mechanisms, several major subdivisions of the phylum remain unexplored, no arachnid, for example, having been examined in regard to its detailed neuromuscular mechanisms. It seems desirable, therefore, to have information regarding the motor mechanisms of the particularly interesting primitive arachnids, the Xiphosura. Accordingly a preliminary study has been made on *Limulus polyphemus* Latr. and has revealed several interesting features which are reported here.

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METHODS

The walking legs, except the specialized fifth pair, have been examined from specimens 16″–22″ long, obtained at Woods Hole, with a view to finding suitable nerve-muscle preparations. The legs were severed by a quick snip of the coxo-trochanteral joint. A few of the leg muscles can be used, in particular the closer of the claw (adductor or depressor of the tarsus) and the flexor (levator) tibiae (situated in the patella). The present studies were carried out entirely on the claw closer. This muscle exhibits in the freshly-excised leg a remarkable pseudo-reflex. If the inside of the pollex (fixed extension of the tibia) is gently stroked, the claw closes sharply. This reflex can be obtained repeatedly for up to 15 minutes or so after removal of the leg. It seems highly improbable that any nervous machinery of true synaptic type can be present in the isolated leg to account for this curious phenomenon. Similar phenomena have been described in excised crustacean legs in which stretching of the chela, for example, can lead to its opening. Wiersma (unpublished) has suggested as an explanation of the crustacean responses that following excision the excitability of the cut ends of the motor nerves is raised to such an extent that an ephaptic transmission occurs from adjacent sensory axons.

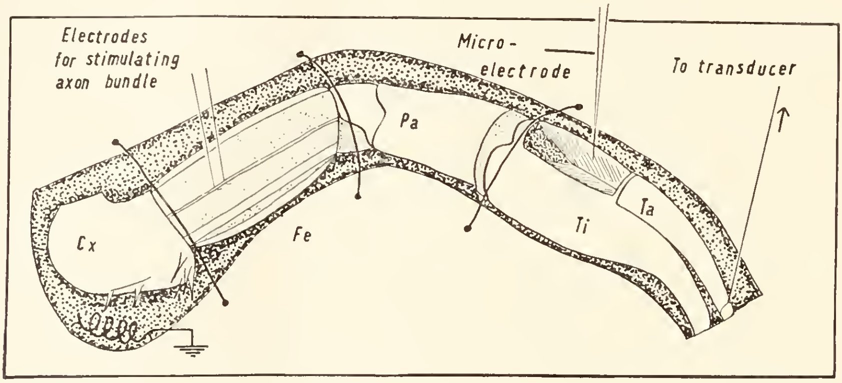

FIGURE 1. Drawing of the preparation seen from above. The leg is placed in a trough cut in a wax block. The opener of the claw has been removed, exposing the closer muscle.

To make a preparation, the excised leg was laid in a sculptured trough of wax and stapled into position with the tarsus uppermost. The main leg nerve can then be easily exposed in the femur by cutting away the shell and removing part of the extensor patella muscle. The nerve has no surrounding sheath and very little connective tissue so that it can easily be split into bundles. These may be stimulated in turn and any having an effect on the tarsus retained, the rest being cut away. The retained bundles can then be split again until either very small bundles, or eventually single axons, remain.

In this way it was ascertained that the closers of the claws of legs I–IV are innervated by two motor axons. No inhibitory axons were found. In this respect *Limulus* resembles the insects rather than the crustaceans.

There is no tested physiological saline for *Limulus* so filtered sea water was used to bathe the preparation. Cole (1940) has analyzed the haemolymph and found that the mineral composition approximates very closely indeed that of the local sea water in two different localities, one of which was Woods Hole. Since the present work was done, a physiological saline has been developed for the Japanese horseshoe crab, *Tachypleus tridentatus* (Kikuchi and Tanaka, 1957).

At this stage a strip of shell was carefully snipped away from the margin of the tibia in order to expose the outer edge of the opener muscle (abductor tarsi). The opener apodeme was then cut close to the tarsus, grasped with forceps, lifted and stretched until the whole muscle came away. This leaves the V-shaped closer muscle exposed, with its innervation intact.

The pollex was fixed in a hole in the wax block and the tip of the tarsus was attached by a thread to an electromechanical transducer. Pairs of fine silver wires were micromanipulated onto the exposed nerve bundles. A drawing of the preparation, seen from above, is presented in Figure 1.

The muscle fibers are of fairly uniform diameter but only 25–40 μ thick, *i.e.*, they are appreciably thinner than many insect muscle fibers and very much thinner than those of the larger decapod crustaceans. Glass capillary micro-electrodes, filled with 3 *M* KCl, were used to record trans-membrane potentials from muscle fibers of the claw closer. The nerve bundles were stimulated with brief rectangular pulses isolated by radiofrequency coupling units. Display was conventional.

RESULTS

In the more vigorous preparations a single stimulus applied to either of the two nerve fibers evokes in each case a small twitch. Repetitive stimuli lead to partial and complete tetani. The mechanical response to one of the two axons is, however, always larger than the other, and at a given frequency of stimulation also appears slower. Hence the two axons may be referred to as "fast" and "slow" according to the nature of the contraction evoked, as is customary in deal-

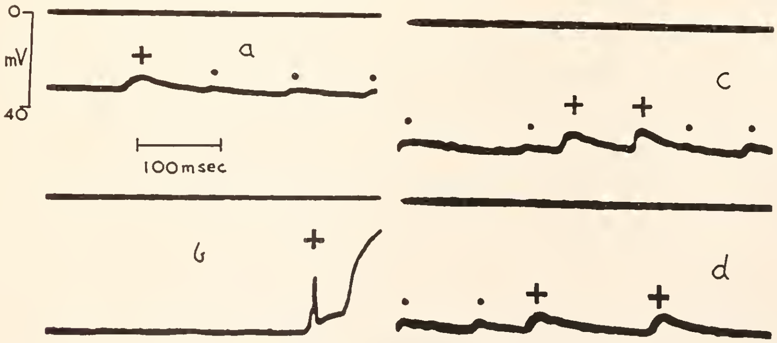

FIGURE 2. "Spontaneous" potentials. Four records from claw-closer muscle fibers of a fresh-excised *Limulus* leg, showing recurring potentials. The deflections marked + are attributed to discharges in the fast axon; those marked · to the slow axon. The single spike response in b was associated with a twitch which must have caused the electrode to be jerked out of the fiber. The upper trace in each record marks the zero baseline and the lower one the internal potential recorded with a 3 *M* KCl-filled glass capillary micro-electrode.

ing with crustacean motor nerve fibers (Wiersma, 1941). The corresponding responses are then called fast and slow, respectively.

"Spontaneous" responses. When the preparation is very fresh, discharges originating in the hypersensitive cut ends of the axons lead to spontaneous "tone" in the closer muscle and contractions which cause small movements of the tarsus. If a micro-electrode is inserted at random into a muscle fiber of the closer at this time, recurring electrical potentials of small size are seen (Fig. 2). The resting potentials of the muscle fibers are of small magnitude, ranging from 35–55 mV. The peak amplitudes of the recurring potentials are from 0.5 mV to a maximum of 25 mV in different fibers. In any one fiber they are clearly of two distinct sizes, the smaller being due to the slow axon and the larger to the fast. Single small potentials are not usually associated with visible twitches although in the more vigorous preparations, when they occurred singly, this was the case, and twitches were seen. A small proportion of muscle fibers gave "fast" potentials which were compound, *i.e.*, they had an initial component resembling an ordinary end-plate-potential (e.p.p.) giving rise to a small spike response (Fig. 2b).

The slow responses. Responses attributable to the "slow" axon could be observed in about 60% of those muscle fibers in which any appreciable electrical change could be obtained during stimulation of both fast and slow axons (usually the bundles containing them) together. The single electrical response was always a very small one resembling a small e.p.p. It will be referred to as a junctional potential (j.p.) rather than an e.p.p. since nothing is known of the nature of the nerve terminals in *Limulus* muscle. To distinguish it from the corresponding response to the "fast" axon it will be called a slow junctional potential (s.j.p.) The long latency following the stimulus artifact, which is apparent in the records, is due largely to the conduction time of the nerve impulse along the nerve in the femur and patella into the tibia.

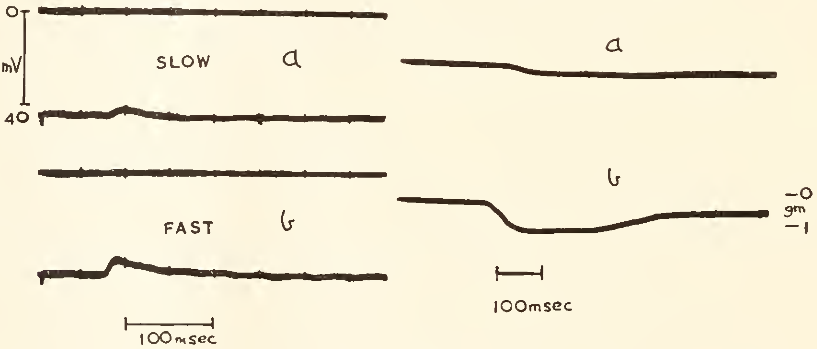

FIGURE 3. Potentials and tension due to single excitations applied to: a, the slow axon and b, the fast axon. Left hand side: electrical responses from the same single muscle fiber. Right hand side: mechanical responses of whole muscle recorded at tarsal tip.

The s.j.p.'s rise to a peak in 12–18 msec. and decay in about 60 msec. The largest one found had a peak amplitude of 5 mV. Although no tension was usually recorded at the tip of the tarsus during stimulation of the slow axon with a single shock, some preparations did show a small twitch, giving not more than

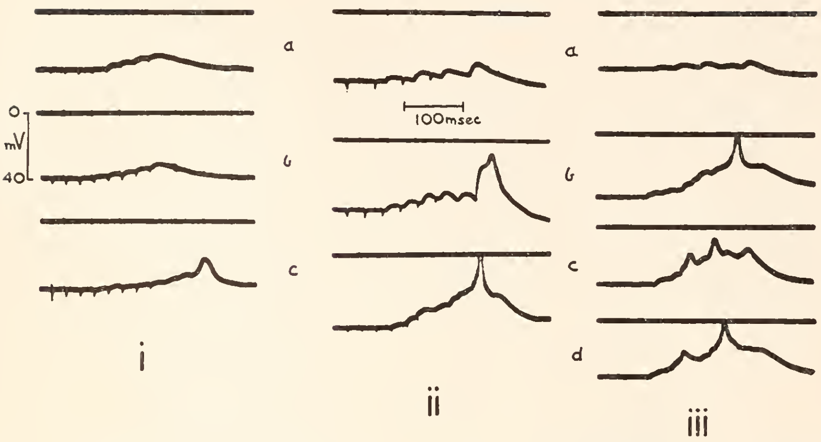

Figure 4. The responses to short trains of stimuli in three different muscle fibers (i–iii). (i) Slow axon. Three responses from the same fiber showing the summation of s.j.p.'s and the small degree of facilitation. A small spike arises in c from the plateau of depolarization. (ii and iii) Fast axon. a, low frequency; b–d, higher frequency. Successive steps (f.j.p.'s) are progressively larger (facilitation). Summation is evident; occasional spikes arise from the depolarization plateau.

0.5 gm. tension at the tip of the tibia (Fig. 3). On repetitive stimulation appreciable tension developed, increasing with increasing frequency of stimulation up to a maximum of just over 50 gm. at 200 per second. Thus the tetanus/twitch ratio was more than 100: 1. The s.j.p.'s initially increased in magnitude by two or three times during a train of stimulation, a phenomenon usually referred to as facilitation, but later diminished as they also summated to give a plateau of depolarization. From the plateau occasionally a small spike arises (Fig. 4i, c).

The fast response. The fast axon evoked electrical responses in most of the muscle fibers penetrated. They were often very small, but they were always larger

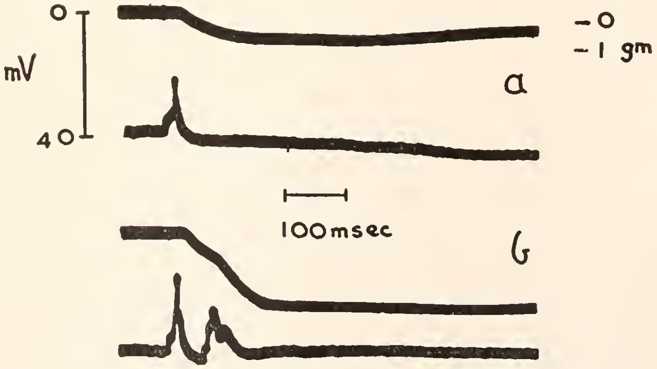

Figure 5. Electrical response of one fiber and total mechanical response at tip of tarsus to: single (a) and paired (b) stimulation of the fast axon. Note that the f.j.p. is followed by a small spike in each case.

than the corresponding slow responses, when these were seen, in the same fibers. There was no overlap of s.j.p. and f.j.p. magnitudes in individual fibers, such as was found in several muscles of decapod crustaceans (Hoyle and Wiersma, 1958a). The typical response to a single shock is shown in Figure 2. The response, like the s.j.p., is of end-plate-potential type and will be referred to as the fast junctional potential (f.j.p.). The rise-time of the f.j.p.'s was usually about the same as that of the s.j.p.'s, *i.e.*, 12–18 msec. and the decay likewise about 60 msec. But occasionally an f.j.p. had a faster rise-time of only 5–6 msec. and/or a faster decay of about 40 msec. In some fibers the f.j.p. leads to a small spike of 10–15 mV. The larger f.j.p.'s reached a peak amplitude of 11 mV.

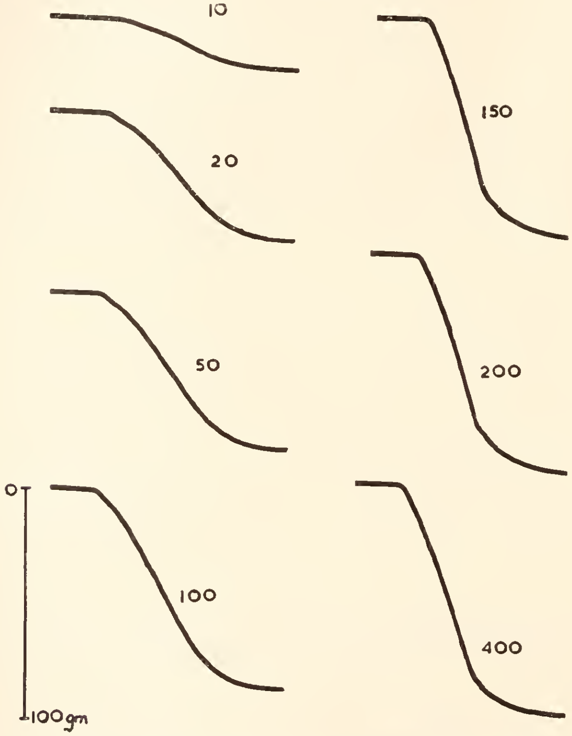

FIGURE 6. Tension recorded during stimulation of the fast axon to show the development of tetanus at the various frequencies indicated.

When pairs of shocks are applied to the axon the mechanical responses summate and also show facilitation (Fig. 5) as the interval between the shocks is reduced. If the first f.j.p. evokes a spike then the second one, at intervals up to 200 msec., seldom does so or gives a much smaller one, *i.e.*, there is a long relatively refractory period for the spike. If the first f.j.p. is of relatively large size but does not give a spike then the second usually evokes one. When the paired shocks are applied regularly, at a low repetition rate, the character of the response is seen to change from time to time. Thus the first f.j.p. will soon fail to evoke a spike but the second will lead to one and vice versa, the process being reversed again after a while. The spike mechanism either fatigues very easily or it is a very labile response.

With prolonged repetitive stimulation, whether there is spiking or not, a plateau of depolarization builds up and is maintained. Brief bursts of stimulation illustrate the way in which the plateau builds up (Fig. 4ii, b–d). At the higher frequencies spikes, taking off from the depolarization plateau, may just reach and occasionally overshoot the zero baseline (Fig. 4i, c). The total tetanus tension and also the rate of rise of tension, continue to increase with increasing frequency of stimulation up to a maximum at about 200 per second (Fig. 6). The tetanus tension measured at the tip of the tarsus then exceeds 100 gm. The tetanus/twitch ratio is ordinarily about 30:1 but it increases as the preparation ages, eventually becoming infinite as the twitch response just fails.

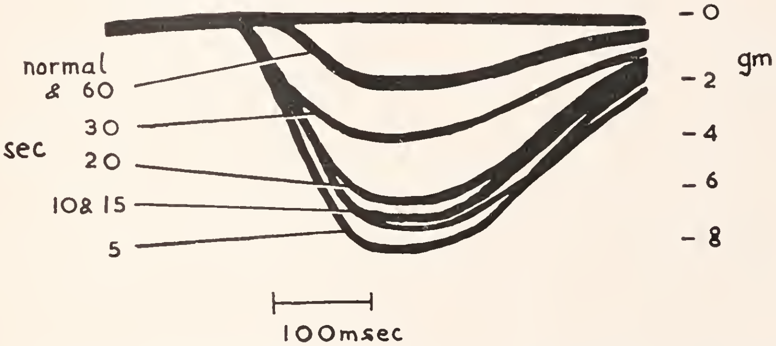

FIGURE 7. Records of tension developed in response to a single shock applied at various intervals (as indicated in seconds) after a brief tetanus (100 shocks at 100/sec.).

The electromechanical transducer used was a fluid potentiometer. When lightly-loaded it recorded the twitch tensions associated with the slow axon of 0.5 gm. and less. Under these loading conditions the tension records did not have the usual shape for a twitch but instead showed a plateau of tension. This may have been caused in part by sluggishness of the potentiometer, but the plateau was too long to be due entirely to this. Thus, in the absence of a large restoring force (the twitch is normal in appearance with a spring attached to the load), tension is maintained for about half a second, after which relaxation occurs.

Post-tetanic potentiation. Following a very brief tetanus there is an enormous potentiation of the twitch response which is regularly 5 times, and may be as much as 7 times, greater than the normal twitch tension. The effect subsides gradually over a period of 45–60 seconds (Fig. 7). The intracellular leads showed no electrical concomitant of this enhancement in the individual muscle fibers examined. It would of course be necessary to examine a large number, particularly in respect to increased tendency to give spikes, in order to be sure that there was no significant electrical effect and this has not been attempted. In the fibers examined the f.j.p.'s were facilitated following the tetanus but only for a few seconds, a fraction of the time during which the tension is potentiated.

DISCUSSION

From the electrical activity recorded in various muscle fibers of the closer of the claw of the walking leg of *Limulus* it may be inferred that the pattern of innervation is substantially similar to that found in doubly-innervated crustacean muscles and non-specialized insect muscles. That is, most of the muscle fibers are themselves innervated by both slow and fast motor axons (polyneural innervation). It has not been established in this investigation that the innervation is also multi-terminal, as it is in those insects and crustaceans which have been examined closely, *i.e.*, that the axons make synapse with the muscle fibers at several points along their length.

The rather low resting potentials and small size of the electrical responses might suggest that the muscles deteriorate following excision of the limb. But for periods up to two hours in which the preparation was used there usually was no (further?) decline in their value. Thereafter, decline was fairly rapid. Tetanus tension measured at the tarsal tip in the preparation is at least as great as that which can be obtained by evoking reflex closure of the claw in the intact animal.

Both the s.j.p.'s and the f.j.p.'s differ in peak amplitude in different fibers although the fast is always larger than the slow. Their rise and decay times have not been determined critically in the present experiments, partly because they were somewhat variable. In some fibers the fast response had both a faster rise time and a faster decay time than the slow, but this was not often encountered and in most cases they had similar values. There was no evidence of a "paradox" situation similar to that found in certain Crustacea (Hoyle and Wiersma, 1958b); *i.e.*, the slow axon did not give tension at lower frequencies of excitation than those which just failed for the fast. There was, in fact, unlike the situation in many crustaceans (Hoyle and Wiersma, 1958a) nothing to indicate that the slow and fast transmitter substances need be regarded as qualitatively different chemically. The preliminary results could be interpreted on the basis of quantitatively different extents of release of one transmitter substance from the terminals of the fast and slow axons.

Each junctional potential attains a constant height over long periods of intermittent stimulation, but the secondary, small spike responses are extremely unpredictable in their appearance and magnitude. They arise only from the larger f.j.p.'s or from the plateaux of depolarization in tetanus. But they cannot be said to appear at a particular level of membrane potential. They may be present on one occasion and absent on the next although the same j.p. deflection is reached in both. Also, they occur randomly, not synchronously, in the population of fibers so that it cannot be determined whether or not their appearance leads to extra tension.

Facilitation of junctional potentials is present in both fast and slow systems, quite markedly in some fibers, hardly at all in others. It is more marked than it appears in the records. The long time-course ensures that there is summation even at low frequencies of stimulation. Hence the later j.p.'s in a train appear at lower and lower levels of membrane potential. Since the magnitude of a j.p. is proportional to the magnitude of the membrane potential, they thus appear quite a bit smaller than they would if the same amount of transmitter action occurred at the normal resting potential level.

The total tension is related to the extent of maintained depolarization, in the randomly-selected muscle fibers studied, during tetanus at different frequencies. But the enhanced tension which occurs in the period following shortly after a tetanus is not reflected in increased depolarization. This argues against there being a simple causal connection between membrane potential and tension. There is probably a connection between the strong post-tetanic potentiation and the fact that there is a high tetanus/twitch ratio, but both must be attributed to intra-muscle-fiber events rather than to neuromuscular junctional ones. All these facts make it seem probable that further and more detailed investigations of neuromuscular transmission in *Limulus* will make valuable contributions to our understanding of excitation-contraction coupling in muscle.

I wish to thank Professor H. Grundfest for the generous facilities which he placed at my disposal in his laboratory at Woods Hole.

Summary

1. The electrical responses occurring in single muscle fibers of the closer muscles of the chelae of the walking legs of *Limulus* have been studied with the aid of intracellular electrodes and electrical stimulation of the motor axons. At the same time the total tension of the muscle was recorded at the tarsal tip.

2. The muscle is supplied by only two motor nerve fibers, one of which (the "fast" axon) evokes larger mechanical and electrical responses than does the other (the "slow" axon).

3. No inhibitory nerve fiber was found.

4. The electrical responses consist typically of junctional potentials resembling small end-plate potentials. The fast junctional potentials may give rise to small spike potentials.

5. On repetitive stimulation both axons give rise to plateaux of depolarization, from which small spikes may arise.

6. The mechanical responses consist of very small twitches to single shocks and tetani to repetitive excitation. The tetanus/twitch ratio is more than 30 : 1 for the fast axon, more than 100 : 1 for the slow axon.

7. There is post-tetanic potentiation of the twitch response of up to 5 times in the mechanical response to a single shock applied to the fast axon. This decays slowly over a period of about a minute.

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THE TOXICITY OF PHYSALIA NEMATOCYSTS[1]

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The siphonophore *Physalia physalis* (Portuguese-man-of-war) possesses a well-merited evil reputation throughout its geographical range. Contact with the tentacles of this animal is always painful to man and may result in vasomotor dysfunction and collapse. Although toxic substances have been previously isolated from *Physalia* tentacles (Richet and Portier, 1936), there appears to have been no examination of the toxic compounds which originate specifically within the nematocyst.

Phillips (1956) described a modification of the method of Glaser and Sparrow (1909) by which the nematocysts of *Metridium* could be isolated, washed and discharged into distilled water. The methods presented here are similar to his. It is our object to present details of the separation of nematocysts from *Physalia* and preliminary data on the composition and toxicity of the separated components. Although *Physalia* is a colonial form, for simplicity, members of the colony will be referred to as if they were anatomical parts and the whole colony as a single entity.

MATERIALS

Physalia appears on southeast Florida beaches during periods of prolonged on-shore winds of greater than usual intensity. Locally these winds may be expected seasonally, from October through March. Small animals generally appear early in the season. Specimens of *Uca pugilator* were purchased from a commercial distributor in the vicinity of Englewood, Florida.

METHODS

Specimens were collected as they stranded and were placed in clean sea water to remove sand. The fishing tentacles were removed, combined with the tentacles from other animals, and allowed to autolyze at 4° C. for 24 to 48 hours. Then the mixture was diluted with one or more volumes of sea water and put through graded screens of 24 and 115 meshes per inch. This removed most of the muscle and connective tissue of the tentacle and permitted passage of undischarged nematocysts. The screened suspension was allowed to settle overnight in the cold. The supernatant solution was decanted and discarded. The residue, which was composed chiefly of nematocysts, was centrifuged at 300–400 g for 15 to 30 minutes. The supernatant solution was again discarded and the residue re-suspended in sea water. These processes were continued until injection of 0.1 ml. of the supernatant solution into the hemocoele of the fiddler crab, *Uca*, was without apparent

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[1] Contribution number 210 from the Marine Laboratory, University of Miami. These studies were supported by U.S.P.H.S. Grant RG-5272.

effect. The nematocyst suspension at this time (Fig. 1) was almost completely free of tentacular tissue fragments and contained approximately 55 million nematocysts per wet gram, very few of them discharged. Nematocysts ranged in size from 8.8 to 42.3 micra. They fell into two size groups: one with a mean diameter of 11.3 micra made up 23% of the total sample. The remainder varied about a mean diameter of 26.8 micra. The packed nematocysts were frozen and stored at − 5° C. Nematocysts were still reactive after 20 weeks of frozen storage. An initial sample of 3.4 liters of isolated fishing tentacles yielded 60 grams of packed wet "purified" nematocysts.

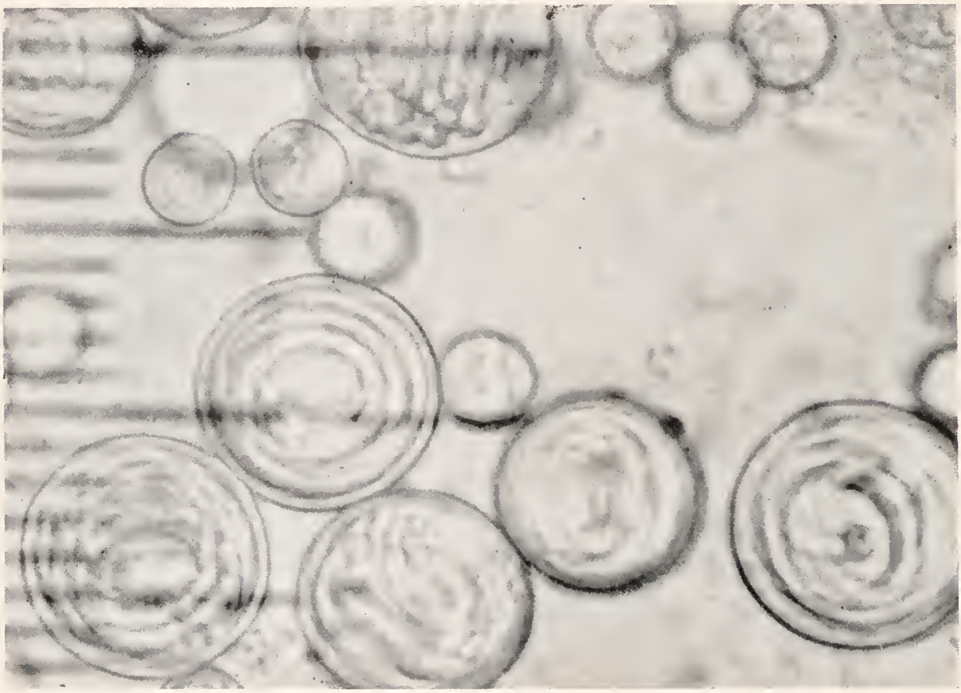

FIGURE 1. Photomicrograph of isolated, purified, still-reactive nematocysts of *Physalia*. × 1400.

The contents of the isolated nematocysts were liberated by homogenization in a chilled Potter-Elvejhem homogenizer. Amphibian Ringer's, sea water or distilled water may be used as the diluent. The sample was examined microscopically at intervals and homogenization was continued until about 90% of the capsules were fragmented (Fig. 2). The homogenate was centrifuged at 600 g for ten minutes to separate capsules and capsular fragments from the diluted capsular contents. The supernatant solution was cloudy, yellowish-white in color, and extremely toxic to crabs, fish and small mammals. Precautions must be taken to avoid exposure of skin to contamination by any mixture, wet or dry, which contains undischarged nematocysts. Nematocysts on laboratory surfaces or clothing retain their reactivity for at least two weeks as unpleasant reminders of previous carelessness. Nematocysts on the tentacles of large living *Physalia* may occasionally penetrate heavy-gauge surgical gloves. Surfaces, clothing and skin can be decontaminated by the application of 95% ethanol. Although this treatment does not reduce the pain of stings already received, it appears to prevent the discharge of additional nematocysts.

Fiddler crabs (*Uca pugilator*) have been employed for initial screening of toxic extracts. Doses of 0.1 ml. of material to be assayed were injected into the hemocoele through the articular membrane of the third walking leg. When sea water alone was administered by this route no effect was produced, but when capsular contents were present in the sea water, paralysis and death ensued. Ten animals were routinely injected with each extract to be assayed. Other animals employed in toxicity determination and evaluation include several species of fish, the frog, and the heart of the clam *Mercenaria campechiensis* (frequently designated *Venus mercenaria*). Acute toxicity studies were done on 30-gram, male Swiss mice, according to the method of Deichmann and LeBlanc (1943).

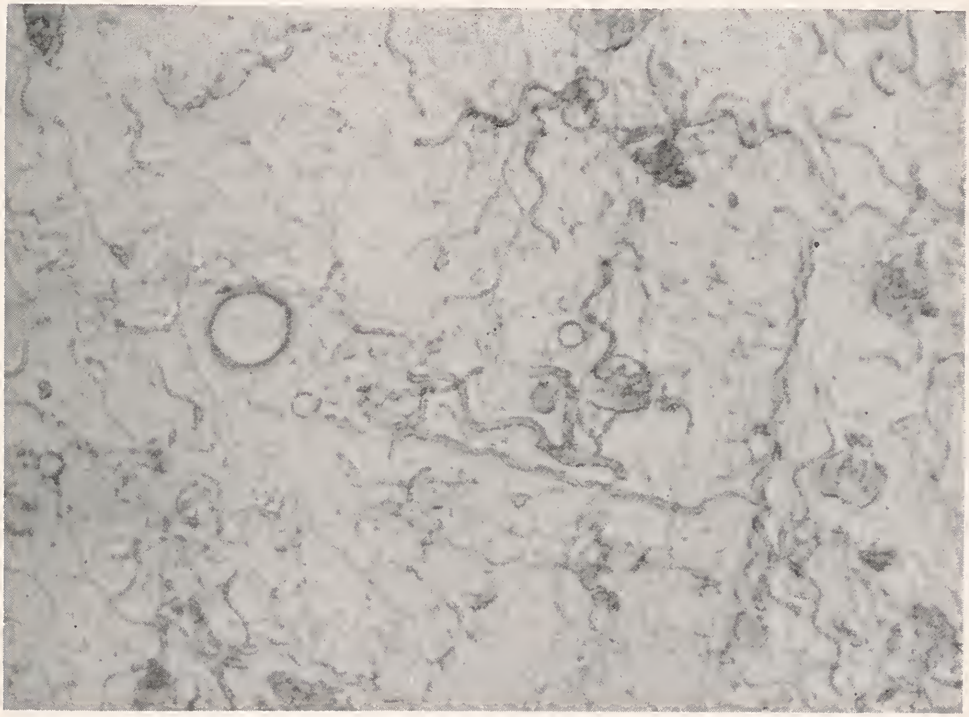

FIGURE 2. Photomicrograph of homogenized nematocysts, × 250. Fragments of capsules, everted tubules and tubule fragments constitute most of the visible formed elements. At least 90% of the capsules have been discharged.

Total nitrogen was determined by the micro-Kjeldahl method; moisture by co-distillation with toluene; and ash by incineration. Dry samples of known weight were hydrolyzed in 6 N HCl in a sealed capsule at 100° C. for 24 to 36 hours. The amino acid content of the neutralized hydrolysate was determined by two-dimensional chromatography on Whatman No. 1 paper. Two-dimensional chromatograms employed n-butanol, acetic acid and water (4 : 1 : 5) as the first solvent and water-saturated phenol as the second solvent. Chromatograms were developed in 0.2% ninhydrin in acetone. The approximate concentrations of amino acids in various samples were estimated by size and density of the amino acid spots on the finished chromatograms.

RESULTS

The distribution of amino acids in *Physalia*, and their approximate concentrations are shown in Table II. The predominant amino acids in undischarged

TABLE I

Composition of entire colonies and of component parts of Physalia

Material	Moisture	Fat	Solids	Ash	% Total N
Entire, living *Physalia*	82.2%	0.23%	17.5%		
Floats, only	88.5		11.48		10.94%*
Fishing tentacles	88.07		11.93		
Gonozooids	88.78		11.22		
Undischarged nematocysts**	77.8		22.2	3.35	2.58
Capsule contents	97.54		1.46		0.598
Discharged capsules (residue)	88.0		12.0		1.48
First wash of discharged capsules					0.12

* This determination refers to dry material; all other N determinations are wet-weight basis.
** This was a standard preparation numbering 55.2 millions of capsules per milliliter.

TABLE II

Amino acids of Physalia

Amino acid*	Entire Physalia	Fishing tentacle	Undischarged nematocysts	Nematocyst contents	Discharged capsules
Cystine	X	X	X	X	X
Cysteine	X	X	X	X	X
Glutamic acid	XXX	XXX	XXX	XXXX	X
Glycine	X	XX	XX	X	XX
Alanine	X	X	XX	X	XXX
Tyrosine	0	0	0	0	0
Proline	X	X	XX	0	XXX
Hydroxyproline	X	X	X	X	X
Leucine	X	X	X	X	X
Isoleucine	X	X	X	X	X
Methionine	X	X	X	0	X
Lysine	X	X	X	X	0
Threonine	X	0	0	0	0
Aspartic acid	X	X	0	0	0
Histidine	X	XX	0	0	0
Serine	X	X	0	0	0

* X means not more than 2.5 micrograms of amino acid in 50 micrograms dry hydrolysate.
0 = no spot.

nematocyst capsules appear to be glutamic acid, glycine, alanine and proline. Of these, glutamic acid is chiefly a constituent of the fluid contents of the capsule, and the others occur chiefly in the solid components of the capsule wall. Lysine, present in the nematocyst complex in small quantity, apparently also is concerned only with the fluid contents. Aspartic acid, histidine, threonine and serine are present in the intact animal, but are apparently not present in the capsular complex.

When an active extract in sea water was administered to the fiddler crab, the response was immediate and predictable. When returned to the container, the injected crab made a short, abrupt run, stopped precipitately, contracted the extensors of the walking legs vigorously. This made the animal appear to rise on tiptoes. It remained motionless during the imperceptible relaxation which culminated in death. If the crabs were handled after relaxation began, responses were limited to the eyestalks and to very slow movements of the walking legs. The animals appeared to be paralyzed. If legs of "paralyzed" crabs were crushed with a hemostat, the number of legs autotomized was only one-third that observed in uninjected crabs similarly treated.

Activity of the fluid contents of the capsule was markedly decreased by heating to 60° C. for 15 minutes, by precipitation with acetone or by extraction with ether. The toxin was non-dialyzable. It was positive to ninhydrin and negative to Benedict's reagent, both before and after acid hydrolysis. Activity persisted without significant quantitative change for at least two months when stored at $-5°$ C. When the capsule contents were precipitated by alcohol and then assayed on crabs, a qualitative fractionation of the total activity was observed. Before treatment with alcohol the extract produced immediate death of test crabs throughout the effective concentration range. After precipitation in alcohol and re-solution of the precipitate in sea water, or variation of the pH, the lethal response was delayed as much as 24 hours, but the extract produced immediate autotomy of the walking legs. Similarly, adsorption of the toxin on paper and subsequent elution released only the autotomy-producing activity. The residue on the paper, as well as the eluate, remained ninhydrin-positive.

The approximate lethal dose for mice of a toxin sample which contained 0.201% total N was 2.1 ml./kilo. when the material was injected subcutaneously (12 mice) and 0.037 ml./kilo. when it was injected intraperitoneally (23 mice). Subcutaneous injection caused depression after about two hours, and death, apparently due to respiratory failure, occurred 12 to 18 hours after injection. Post-mortem examination of a single mouse immediately after it had stopped breathing showed the heart to be still beating, indicating that death was due primarily to respiratory failure. After intraperitoneal injection there was an immediate onset of intoxication—reminiscent of the almost instantaneous response of the fiddler crab. Symptoms included increased activity and tremors probably due to local irritation. After 10 minutes there were ataxia, decreased muscle tone, flaccid paralysis, slowed and labored breathing, defecation, aphrodisia, marked myosis, cyanosis, anoxic convulsions and death. Survival time was 1 to 48 hours, depending on the dose administered. Post-mortem examination showed the following gross pathology: lungs, blanched; heart, contracted, especially the left ventricle; hemorrhagic edema in the peritoneal cavity; skin of nose and ears very white; cornea, cloudy; colon, no formed stools; urinary bladder, empty.

A dose of 0.5 ml. of crude toxin containing 2.43 μ gm. N per ml. was uniformly lethal when injected into the left ventral lymph sac of each of eight frogs (*Rana pipiens*). Within five minutes the white ventral surface of the frog developed irregular red patches which suggested a localized hemodynamic response if not actual escape of blood cells from the capillaries. Breathing became rapid and shallow. Righting and postural reflexes deteriorated progressively during the first hour. At the time the animal first failed to respond to visual stimulation (about 30 minutes), he could be turned over if stimulation of peripheral end organs were minimized. At this time, spinal reflexes appeared to be normal. After 75 minutes, breathing movements ceased and spinal reflexes disappeared. Electrical stimulation of the sciatic nerve elicited no response at this time, but direct stimulation of the gastrocnemius muscle showed it to be normally reactive. The heart continued to beat for 12 to 24 hours. Large amounts of lymph accumulated subcutaneously in the abdominal area. Viscera were hyperaemic, bladder and intestine, empty and in several instances large amounts of bloody intraperitoneal fluid were observed.

Fish responded immediately to intramuscular injection of lethal doses of crude toxin by hyperventilation and rapid swimming. Petechiae often appeared at the sclero-corneal junction. After five minutes to several hours, depending on dosage, the fish became disoriented, sank to the bottom of the tank, and died after a period of one to four hours. This response is typical of the pilchard *Harengula humeralis*, silversides *Hepsitia stipes* Müller and Troschel, and *Fundulus heteroclitus*. These species exhibited chromatophoric responses to injection, usually blanching at the immediate site of the injection and darkening over the general body surface. Examination of two pilchard which had fallen to the bottom of the tank immobilized, showed the heart of each to be beating normally. No abnormal effects were observed after intraperitoneal injection of a lethal dose of toxin. Washed erythrocytes of the mullet (*Mugil cephalus*) did not hemolyze when incubated at 37° C. with several dilutions of crude toxin.

The heart of the clam *Mercenaria campechiensis*, isolated by the method of Welsh and Taub (1948) responded to administration of crude toxin at a concentration of 5.2 μ gm. N/ml. of bath. The pattern of response was similar to that obtained with acetylcholine, *i.e.*, cessation of beat in diastole. The crude toxin appeared to produce irreversible changes which prevented the heart from giving an equivalent response to a similar dosage later. Administration of the crude toxin did not modify the response of the heart to acetylcholine.

DISCUSSION

The crude toxin of the nematocyst is apparently a protein complex or is associated with a protein. The lethal components obscure secondary or side reactions. After warming to 60° C., adsorption on paper and subsequent elution, precipitation with ethanol and subsequent re-solution, manipulation of pH or other mild treatments, at least two fractions of the total activity were resolved and then exerted separate effects on test animals. Welsh (1956) has shown that many substances, including extracts of *Physalia* and various other coelenterates, modify the autotomy reflex in crustaceans. It is therefore of considerable interest that a compound which caused autotomy appeared in the capsular contents only after this material had been subjected to drying, heating, or other procedures which cause denaturation. This effect has not been produced by unmodified extracts.

Lenhoff, Kline and Hurley (1957) have described a characteristic chemical composition of nematocyst capsules of other coelenterates. They have suggested, together with Phillips (1956), that the capsule is similar in chemical composition to the collagenous group of proteins of higher animals. In homogenized preparations of *Physalia* nematocysts, the capsules tend to retain their general shape though they be ruptured or even broken completely in two. This observation provides a certain amount of support for the concept that the capsule wall is semi-rigid.

Our data suggest that *Physalia* differs from *Metridium* in that the amino acid spectrum of the capsule contents differs both qualitatively and quantitatively from that of the capsule wall. Apparently no hexose constituents are present in *Physalia* although Phillips describes hexoseamines from *Metridium*.

Our methods of isolation of nematocysts of *Physalia* require no other diluent than sea water, with which the nematocysts are presumably normally in contact. This avoids the introduction of extraneous salts and may contribute to the long persistence of reactivity we have observed. We have elected to liberate the capsule contents by homogenization rather than to await the considerable time that may be required for normal discharge. The lability of *Physalia* toxin necessitates a minimum of delay in processing.

Injection of crude toxin apparently produces a general paralysis. It appears to affect the nervous system, especially respiratory centers, before the muscular system. In the frog, the central nervous system is apparently affected before the peripheral nervous system. Crude toxin seems to alter the permeability of capillary walls in mice, fish and frogs. Hemolysis was not observed.

Since the toxicity of the capsule contents of *Physalia* is reduced by some organic solvents, and since these solvents also inactivate adherent nematocysts, the local application of alcohol to the skin of a swimmer stung by *Physalia* is an effective palliative measure.

SUMMARY AND CONCLUSIONS

The general composition and conditions of reactivity of the nematocysts and nematocyst contents of *Physalia* are described. A method is presented for isolation of nematocysts without contamination by other tentacular material. The nematocyst content appears to be a highly labile protein complex. The toxicity of the capsule contents is destroyed or denatured by heating to 60° C., by drying, by treatment with ethyl ether, acetone, or ethanol. Activity may be preserved for two months when the material is stored at − 5° C. The approximate lethal dose for mice, when the toxin was injected intraperitoneally, was 0.037 ml./kilo. of a preparation which contained 0.201% total N. The toxin was shown to be devoid of hemolytic activity for fish erythrocytes. When tested in fish, frogs or mice it appeared to affect the nervous system, particularly the respiratory centers, before voluntary muscles. Localized changes in cardiovascular tone have been observed in some test animals. *Physalia* toxin elicited responses in the isolated heart of the clam which were similar to those caused by acetylcholine.

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ON THE EVOLUTION OF HEMOGLOBIN. RESPIRATORY PROPERTIES OF THE HEMOGLOBIN OF THE CALIFORNIA HAGFISH, POLISTOTREMA STOUTI[1]

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The Cyclostomata, which is composed of the hagfishes (Myxinoidia) and the lampreys (Petromyzontia), is considered on morphological (Young, 1950) and biochemical (Florkin, 1949; Wald, 1952) evidence to be the most primitive group of living craniate vertebrates. Whereas the molecular weight of vascular hemoglobin of all known non-cyclostome vertebrates corresponds to four oxygen-combining units (hemes) per molecule, the hemoglobin of both hagfishes and lampreys consists of but a single heme per molecule (Svedberg, 1933; Lenhert, Lowe and Carlson, 1956). With regard to amino acid composition, cyclostome hemoglobin appears to be intermediate between vertebrate and invertebrate hemoglobins (Florkin, 1949).

The oxygen equilibrium of hemoglobin solutions prepared from the blood of the sea lamprey, *Petromyzon marinus*, has been recently studied (Wald and Riggs, 1951). This hemoglobin possesses a hyperbolic oxygen dissociation curve (as would be expected on the basis of the above-mentioned molecular weight), a low oxygen affinity, and an extremely large Bohr effect. Wald (1952) has claimed that the evolution of hemoglobin has proceeded in three stages (p. 366): "(1) the heme enzymes of cellular respiration [cytochrome oxidase being considered as the phylogenetic precursor of hemoglobin (Wald and Allen, 1957)]; (2) cell and tissue hemoglobins concerned primarily with oxygen storage; and, (3) circulatory hemoglobins, concerned with the transport of oxygen from the lungs, gills, and skin to the internal tissues." Wald emphasizes that in this progression the three main biochemical aspects of the combination of hemoglobin with oxygen are altered: (1) the oxygen dissociation curve changes from hyperbolic to sigmoid—*i.e.*, heme-heme interaction develops; (2) the affinity for oxygen decreases—*i.e.*, the oxygen molecule is held less tightly to the heme; and (3) the oxygen affinity becomes a function of pH—*i.e.*, a Bohr effect is developed.

In view of these facts concerning the cyclostomes, and Wald's (1952) theory on the evolution of hemoglobin, it is of interest to evaluate the oxygen equilibrium of the hemoglobin of the California hagfish, which is perhaps an even more primitive vertebrate than the lamprey.

The author wishes to thank Denis L. Fox and the other members of the Division of Marine Biology for their assistance in and discussion of these studies,

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[1] Contribution from the Scripps Institution of Oceanography. New Series.

and David Jensen for provision of hagfish and for assistance in bleeding of specimens.

MATERIALS AND METHODS

Blood was obtained from 30 specimens of the California hagfish, *Polistotrema stouti* (Lockington), formerly called *Bdellostoma* or *Eptatretus stouti*. The blood was collected by placing capillary tubes adjacent to the severed ends of blood vessels; no anticoagulant was necessary, for the blood of this animal has little clotting ability. The blood was occasionally contaminated with a trace of the ubiquitous slime; this was easily removed by diluting the blood with isotonic phosphate-buffered saline and filtering through glass wool. In several cases blood from a single animal provided enough hemoglobin for a single oxygen equilibrium determination. However, the hemoglobin concentration is low (3–4%), and usually less than 1 cc. of blood is available from each animal; therefore, blood from several animals was often pooled. Erythrocytes were either (1) washed once in 15 cc. of isotonic saline and used immediately for determination of the oxygen equilibrium of dilute erythrocyte suspensions (equivalent to whole blood), or (2) washed two more times and then hemolyzed. Distilled water hemolysis did not give satisfactory results; up to 80% of the hemoglobin remained inside the cell. Therefore, a trace of powdered saponin was added to a suspension of one volume of cells to two volumes of distilled water. Several hours later the hemoglobin solution was separated as a supernatant by centrifugation, diluted with an equal volume of potassium phosphate buffer ($\Gamma/2 = 0.4$) of the desired pH, and then filtered through Whatman No. 5 paper. Such a hemoglobin solution is stable for days, although (except where specifically indicated) it was used immediately for oxygen equilibrium measurements. Preparation of hemoglobin was at 0–1° C., except for centrifugation at 8–12° C.

Oxygen equilibria were evaluated as in previous studies (Manwell, 1958a, 1958b). Erythrocytes were suspended in 9 parts isotonic sodium chloride (0.54 *M*) to 1 part potassium phosphate buffer of desired pH. To eliminate rapid settling of cells during spectrophotometric determination of oxyhemoglobin, and to reduce light-scattering effects, many erythrocyte suspensions were diluted 3 : 1 with Karo (a mixture of sugars, dextrins, and soluble starch, which has a high refractive index and thus effects a partial clarification of the cell suspension). Erythrocytes could be stored for a week in such a medium without hemolysis, although this undesirable effect took place to a slight extent in a few experiments involving prolonged equilibration. Therefore, some experiments were performed on cells simply suspended in buffered saline to which a trace of powdered bovine serum albumin was added to increase cell stability; in these instances absolutely no hemolysis was observed during or for a day after equilibrium measurements, although there was greater fluctuation in spectrophotometric readings due to settling of cells and rouleaux.

Most experiments were performed at 18° C., slightly above the upper limit of the physiological temperature range of the hagfish. However, in connection with a determination of the heat of oxygenation of this hemoglobin some studies were made at 11° C., well within the normal temperature range, and at 29–30° C.

RESULTS

Instead of presenting all data in the form of the usual "oxygen dissociation curve," the linear transformation based on the Hill approximation,

$$y = 100 \frac{(p/p_{50})^n}{1 + (p/p_{50})^n},$$

is used in Figures 1 and 3 (Lemberg and Legge, 1949). The variables y and p are the per cent oxyhemoglobin and the partial pressure of oxygen, respectively.

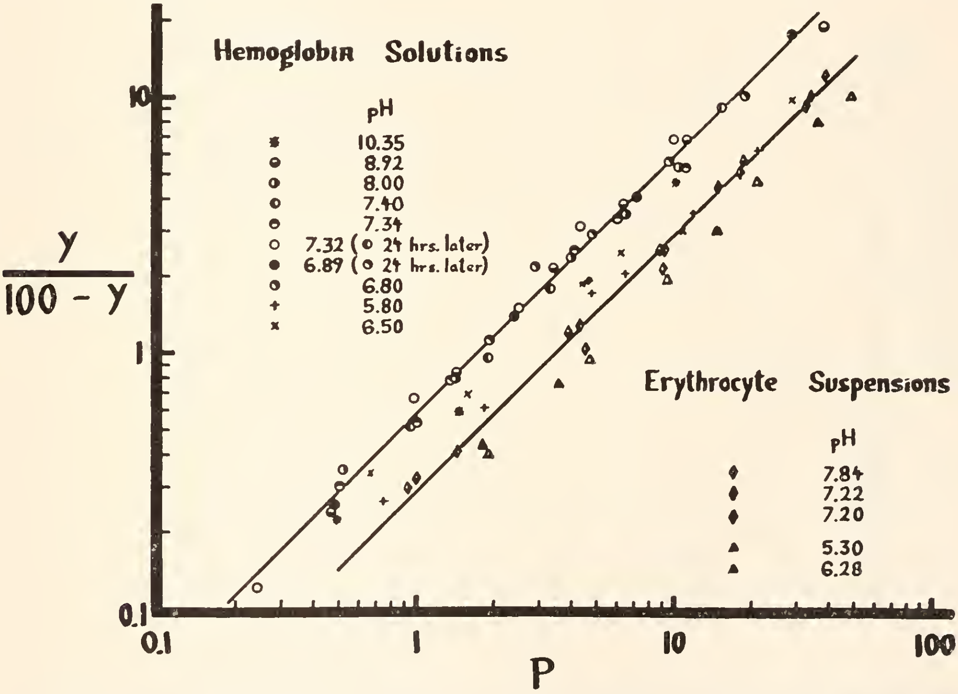

FIGURE 1. Oxygen equilibrium of hemoglobin of the California hagfish, *Polistotrema stouti.* Three to four per cent hemoglobin solutions in potassium phosphate buffer; final ionic strength = 0.2. Erythrocyte suspensions in isotonic phosphate-buffered saline with Karo added as explained in text. Temperature = 18° C. The solid lines are drawn arbitrarily with a slope $(n) = 1.00$ and a p_{50} corresponding to approximately physiological pH's.

That value of p for which y equals 50% is the p_{50}. The "sigmoid coefficient," n, is a measure of the heme-heme interactions. Hence, p_{50} is an inverse measure of the oxygen affinity, and n determines the shape of the oxygen dissociation curve. If the slope of the transformation, $\log [y/(100 - y)]$ as a function of $\log p$, is one, then the hemes are totally independent—*i.e.*, there is no heme-heme interaction. As can be seen from Figures 1 and 3, where the solid lines are drawn with a slope of 1.00, this is true of hagfish hemoglobin inside and outside the erythrocyte, and at high and low temperatures.

Between pH 6.7 and 9.0 hagfish hemoglobin in solution shows no detectable Bohr effect. Outside that pH range a significant decrease in oxygen affinity occurs; however, this effect appears to be a prelude to more drastic changes (methemoglobin formation and decrease in solubility), which become apparent several hours after equilibrium measurements. This is in contrast to the solutions at intermediate pH which are stable for days and display identical oxygen equilibria when re-analyzed one or two days after the original measurements (see Figure 1). No Bohr effect was observed for erythrocyte suspensions at pH's above neutrality; however, paralleling the behavior of hemoglobin in solution, a slight oxygen affinity decrease occurs at acid pH's. The effect was shown not only by the partially clarified suspensions (Fig. 1), but also when no Karo was present (Fig. 2). In contrast to hemoglobin solutions such acidic erythrocyte suspensions were stable, possibly because of the presence of cellular reducing systems able to reduce any methemoglobin. The observed decrease in oxygen affinity could represent a very small Bohr effect; however, until it is shown that the decrease in oxygen affinity is *rapid* and *entirely reversible*, the possibility of slight denaturative changes in the protein cannot be overlooked, especially in view of the results obtained for hemoglobin solutions.

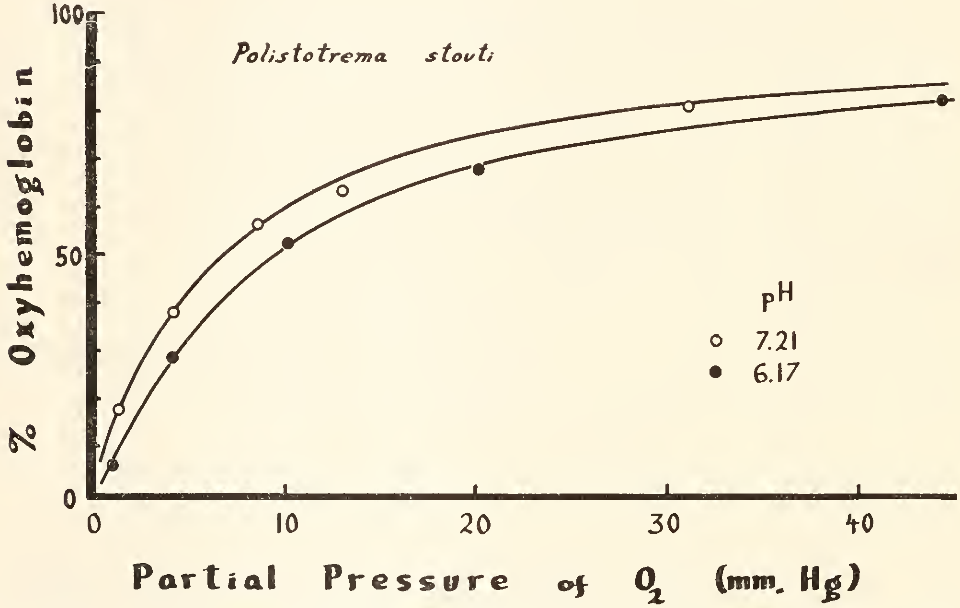

FIGURE 2. Oxygen dissociation curves of erythrocyte suspensions of the California hagfish, *Polistotrema stouti*, at two different pH's, showing the possible very slight Bohr effect. Erythrocytes in phosphate-buffered saline; no Karo present. Temperature = 20–21° C.

The presence of CO_2 specifically decreases the oxygen affinity, in addition to its effect resulting from the increase in acidity, for hemoglobin of the horse (Margaria and Milla, 1955) and the teleost *Sebastodes ruberrimus* (Manwell, unpublished data). That CO_2 does not cause any special Bohr effect for hagfish hemoglobin is shown in Figure 3.

Because the possible Bohr effect of hagfish hemoglobin is so small and occurs at almost one pH unit below the normal pH of hagfish blood (7.5–7.7; Prosser *et al*, 1950; David Jensen, personal communication), it is reasonable to assume that it is of no physiological significance, especially as CO_2 does not have any specific effect.

Knowledge of the heat of oxygenation (ΔH°) of hagfish hemoglobin enables one to predict the position of the oxygen equilibrium at any particular physiological temperature. In addition, absence of a Bohr effect facilitates the evaluation of ΔH°, for corrections representing the effects of ionizations of heme-linked groups do not need to be applied. Using data presented in Figure 3, a value of ΔH° = −9.3 kcal. (one atmosphere of dissolved oxygen gas at the standard state) was obtained as in a previous study (Manwell, 1958a). This value of ΔH° is similar to those observed for hemoglobin of sheep (− 8.2 kcal.; Paul and Roughton, 1951), the holothurian *Cucumaria miniata* (− 8.4 kcal.; Manwell, 1958f), and adult and fetal spiny dogfish, *Squalus sucklcyi* (− 8.7 to − 9.5 kcal.; Manwell, 1958d). This group of values for the heat of oxygenation of various hemoglobins is not

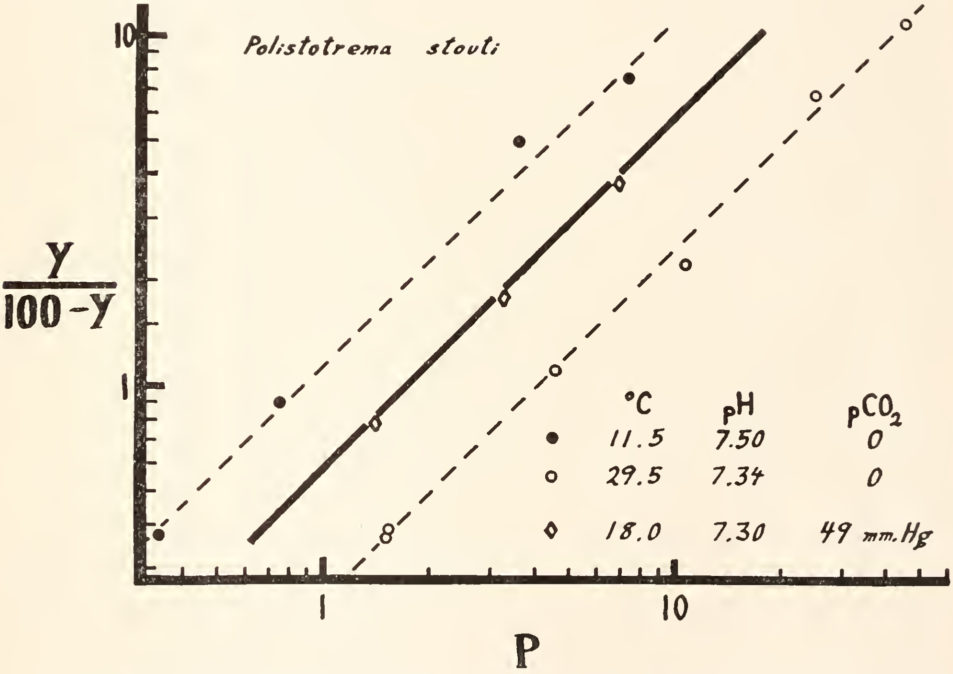

FIGURE 3. Oxygen equilibria under a variety of conditions of hemoglobin solutions prepared from the blood of the California hagfish, *Polistotrema stouti*. Dashed lines are drawn to approximate the oxygen equilibrium at 11.5 and 29.5° C. The solid line is drawn on the basis of data presented in Figure 1 in order to facilitate the comparison of hemoglobin solutions in the presence and in the virtual absence of CO_2.

characterized by the extensive variation seen in older data (reviewed by Paul and Roughton, 1951); theoretical considerations imply that there be relatively little variation in these values for a particular respiratory pigment, although significant differences occur between hemoglobin, hemocyanin, and hemerythrin (Klotz and Klotz, 1955; see, also, Manwell, 1958a).

Discussion

Biochemical and Physiological

Hagfish hemoglobin has a hyperbolic oxygen dissociation curve. Beyond that point, however, resemblance to sea lamprey hemoglobin (Wald and Riggs, 1951) ceases. Hagfish hemoglobin lacks a Bohr effect over a pH range well in excess of pH's to be expected in living hagfish. The hagfish is, accordingly, the first adult vertebrate whose blood is known to lack a Bohr effect. In addition, the oxygen affinity of hagfish blood is very high—at physiological conditions as high, if not higher than that of any known vertebrate blood; the p_{50} is 2–4 mm. Hg [2] over the temperature range of 5–15° C. By way of comparison, p_{50} for human blood is 26 mm. Hg at pH = 7.3–7.4 and a temperature of 37° C. (Prosser *et al.*, 1950). *Arenicola* hemoglobin has an especially high oxygen affinity, $p_{50} = 2$–2.5 mm. Hg at 20° C. (Allen and Wyman, 1952); yet, by virtue of its extremely sigmoid oxygen dissociation curve ($n = 6$) *Arenicola* hemoglobin appears to be more suitable for oxygen transport than hagfish hemoglobin.

The comparison between hemoglobin solutions and erythrocyte suspensions prepared from the blood of the hagfish indicates that no specific interaction occurs between the hemes of adjacent hemoglobin molecules inside the cell. Hence, these data are consistent with—but do not necessarily establish—the idea that the molecular weight of hagfish hemoglobin *in situ* corresponds to but one heme per molecule—*i.e.*, approximately 17,000–18,000.

Comparison of the results with the introduction to this study shows that hagfish hemoglobin possesses all three of the features of the oxygenation reaction considered to be primitive by Wald (1952). However, the properties of hagfish hemoglobin, considered to be characteristic of *storage* hemoglobin by Wald, are displayed by a vascular hemoglobin, which one might accordingly assume to be involved in oxygen *transport*.

Living hagfish have been examined in an attempt to see whether there may be a significant difference in the color of blood entering and leaving the tissues and the gills, a condition which would indicate participation of the hemoglobin in oxygen transport. Unanesthetized hagfish were pinned at the extreme caudal and cranial ends (but not unnaturally stretched out) to a board immersed in oxygenated sea water at 10–12° C. The animals struggled violently until both ends were pinned down; they then remained quiescent for the duration of the experiment. A median ventral incision was made in the vicinity of the liver and the heart, care being taken to avoid cutting any blood vessels. The slime secretions were periodically removed. When slime production ceased, blood in the dorsal aorta (leaving the gills) and in various veins (leaving the tissues) was compared visually with "reduced" and oxygenated standards in hagfish blood vessels. Blood in the veins appeared to be almost de-oxygenated; blood in the arteries was approximately 50% oxygenated. This condition did not change over several hours of continuous observation. By reference to the oxygen dissociation curves, it can be seen that the internal oxygen tensions were extremely low, although the hemoglobin was functional in oxygen transport. An improved physiological experimental approach would be highly desirable; however, the hagfish—considering its small size, its surprisingly violent activity when handled, and its copious slime-producing abilities—is not an especially suitable form in which to determine arterial-venous oxygen concentrations.

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[2] This approximate range of p_{50} for hagfish blood at physiological temperatures has been calculated (Manwell, 1958a) on the basis of the p_{50} for erythrocyte suspensions at 18° C. (see Fig. 1) and the heat of oxygenation ($\Delta H°$) of hagfish hemoglobin in solution.

Wald (1952) comments (p. 367): "The business of a circulatory hemoglobin, having combined with oxygen at the body surface, is to release it in the tissues *at high tensions. . . .*" (Italics are those of Wald.) Clearly, hagfish hemoglobin is biochemically unable to function in such a way; and, the observations made on living specimens tend to strengthen the idea of oxygen transport at *low* internal oxygen tensions in *Polistotrema stouti*. Redmond (1955) has found extensive evidence for oxygen transport at low internal oxygen tensions in several decapod crustaceans. Several studies indicate that such a condition also exists in some but not all annelids (reviewed by Eliassen, 1953; see also, Jones, 1954; Eliassen, 1955; Manwell, 1958e). Adult spiny dogfish, *Squalus suckleyi*, have a hemoglobin with a hyperbolic oxygen dissociation curve inside and outside the erythrocyte (Manwell, 1958d); yet, polarographically determined oxygen tensions of blood leaving the heart were never above 5 mm. Hg in 15 resting dogfish. Very low venous oxygen tensions have been observed in some teleosts—but not the mackerel (Black, 1951). Especially interesting in this regard is the marked *suppression* of heme-heme interaction by the erythrocytes of some teleosts and a species of holocephalian (Manwell, unpublished data); although n for clingfish *Gobiesox* hemoglobin in solution is 2.5–2.6 and thus approaches values of n for mammalian hemoglobins (2.6–3.0), inside the red blood cell the oxygen equilibrium of *Gobiesox* hemoglobin is almost devoid of heme-heme interaction ($n = 1.2$–1.4); this trend is exactly the opposite of what would be expected were the sigmoid oxygen dissociation curve always so vital for oxygen transport.

Under conditions where the tissues tolerate—or require—low oxygen tensions the properties usually associated with a transport hemoglobin would be of little selective advantage. In addition, if a large diffusion gradient were necessary to account for movement of sufficient oxygen across the epithelium of the gills or skin, then such properties as low oxygen affinity and large Bohr effect would prevent loading of the respiratory pigment with sufficient oxygen in the organ of external respiration. ("Sufficient" does not imply *complete* saturation; see Redmond, 1955.) Partial use of anaerobic metabolism could free tissues from dependence on large internal oxygen tension gradients. At the same time as such a rigorous dependence on oxygen were reduced, however, so would the metabolic efficiency decline (aerobic metabolism yielding several times more energy per unit weight of substrate than anaerobic metabolism). Consequently, one might expect large, very active animals (*e.g.*, cephalopods, some fishes, birds, and mammals) to have evolved increasing dependence on the more efficient aerobic metabolic pathways—and at the same time oxygen transport at high internal oxygen tensions. In such cases the sigmoid oxygen dissociation curve, the low oxygen affinity, and the large Bohr effect would be of the greatest selective advantage in increasing the efficiency of the respiratory pigment. It is well known that squid hemocyanin, mammalian and avian hemoglobins, and mackerel and trout hemoglobins possess all of these characteristics (reviewed by Florkin, 1949; Prosser *et al.*, 1950).

Phylogenetic

In terms of Wald's (1952) previously mentioned theory on the origin and evolution of hemoglobin one might be tempted to infer that the primitive hagfish has retained in a hemoglobin used in oxygen transport all three oxygenation properties to be expected of hemoglobin in an earlier stage of evolution—that represented by an oxygen storage hemoglobin. However, some or all of the properties of hagfish hemoglobin may represent specialization to a particular mode of life far different from that of known fossil Agnatha. The hagfishes are, in spite of some primitive characteristics, well-adapted, biologically successful animals. Over several types of ocean bottom in temperate seas the hagfishes are among the dominant scavengers—or parasites—feeding on dead and dying fishes; they are often present in such numbers as to restrict or prevent several types of fishing operations (Young, 1950). Certain characteristics of the hagfish, such as the rasping tongue, complete absence of scales and bone, and the habit of feeding on teleost fishes, are not properties of fossil Agnatha (Ostracoderms). These features must have evolved independently of other aspects of early vertebrate phylogeny. The differences in the properties of sea lamprey (Wald and Riggs, 1951) and hagfish hemoglobin may be correlated with the well-known ecological observation: the hagfish enters, often in large numbers, the body of its prey and thus is often exposed to low O_2 and high CO_2 tensions; the lamprey remains attached to the surface of its host, thereby having well-oxygenated water of low carbon dioxide tension available for its respiration at all times. In addition, so far as is known, the hagfish does not make any sustained active movement comparable to the anadromous migration of the sea lamprey.

Several other objections to Wald's (1952) theory in its present form can be raised:

(1) Cytochrome oxidase has been considered the phylogenetic precursor of hemoglobin because: (a) it combines reversibly with CO and reacts with O_2; and, (b) beef heart cytochrome oxidase has an extremely high oxygen affinity, no Bohr effect, and an almost hyperbolic equilibrium curve with CO—all properties that a "primitive" hemoglobin ought to possess (Wald and Allen, 1957). Unfortunately, neither the prosthetic group (Paul, 1951; Stotz, Morrison and Marinetti, 1956) nor the protein moiety (Lemberg and Legge, 1949) of this respiratory enzyme (or enzyme complex) resembles the corresponding parts of hemoglobin as closely as might be desired. Cytochrome *c* would be a better, although not entirely satisfactory, hemoglobin phylogenetic precursor. At least its prosthetic group is the same as that of hemoglobin, although linked to the protein differently; and, its protein moiety is readily water-soluble, although of lower molecular weight (one heme per 13,000–15,000) and higher isoelectric point (pI = 10) (Paleus, 1955) than any known hemoglobin. When the heme of cytochrome *c* is not completely protected by coordination of the iron with the imidazole groups of two

histidine residues, the enzyme combines with CO and is oxidized by O_2 (Lemberg and Legge, 1949; Theorell, 1956). Bartsch and Kamen (1958) isolated a bacterial heme protein—originally called a "pseudohemoglobin"—which resembles cytochrome *c* in many respects, although its isoelectric point (pI = 5) is comparable to that of invertebrate and cyclostome hemoglobins (Prosser *et al.*, 1950) and it is readily oxidized by O^2 and combines reversibly with CO. The carbon monoxide reaction of this bacterial heme protein is *not* invariant to pH change—in contrast to cytochrome oxidase (Wald and Allen, 1957). In support of *some* connection between the syntheses of cytochrome and hemoglobin is the finding of Ycas (1956) that aerobically grown yeast in the presence of antimycin produces less cytochrome *a* and more hemoglobin than controls; however, as Ycas suggests, this relation may be explained by assuming that the heme of hemoglobin is a precursor to the modified heme of cytochrome *a*. At present there is so little comparative biochemical information on the cytochromes and other heme-containing enzymes that one cannot rule out the possibility that the proteins of various hemoglobins have arisen from apoenzymes of quite unrelated biocatalysts; certainly, the protoheme prosthetic group is always phylogenetically available. Several proteins besides globin will combine with heme, although none are yet known that will enable this heme to combine reversibly with molecular oxygen (Lemberg and Legge, 1949).

(2) Wald (1952) states (p. 369): "The hemoglobins that have arisen so sporadically among invertebrates of various orders are all storage hemoglobins." However, oxygen transport by hemoglobin occurs in several annelids (Johnson, 1942; Eliassen, 1955; reviewed by Eliassen, 1953; Manwell, 1958e), the brine shrimp *Artemia* (Gilchrist, 1954), and even such small arthropods as daphnids (Hoshi, 1957). As the experiments of Redmond (1955) show, the presence of a respiratory pigment in the blood of invertebrates in low concentration does not rule out significant oxygen transport by that pigment. Coelomic hemoglobins, such as those of *Urechis* (Redfield and Florkin, 1931) and *Cucumaria miniata* (Manwell, 1958f), are usually assumed to function in oxygen storage; however, the movement of coelomic fluid, either by muscular contraction or cilia, presents the possibility of oxygen transport by the coelomic hemoglobin from cloacal diverticula (*Urechis*) or respiratory trees (*Cucumaria*) to tissues in or adjacent to the coelom.

(3) That a hyperbolic oxygen dissociation curve, high oxygen affinity, and no Bohr effect should represent primitive conditions (Wald, 1952) requires comment. The properties of the oxygen equilibrium of the vertebrate storage hemoglobin (myoglobin) rest on studies of crude extracts or purified preparations prepared from the muscles of five species of mammals (reviewed by Lemberg and Legge, 1949; see also, Rossi-Fanelli and Antonini, 1958). As Lemberg and Legge point out, the oxygen equilibrium of myoglobin *in situ* in the muscle remains to be evaluated. The findings, that *n* could be as high as 1.6 for oxygen equilibria of extractions of *Cryptochiton* myoglobin (Manwell, 1958c) and that *n* could be as high as 2.8 in the reaction of horse metmyoglobin with various ligands (Kiese and Kaeske, 1942), indicate that heme-heme interactions can exist under certain conditions in tissue hemoglobins. One would expect tissue hemoglobins to have a high oxygen affinity because of limitations on the intracellular oxygen tensions imposed by the combination of passive diffusion of oxygen and aerobic cellular

metabolism. In the case of *Cryptochiton* even when the oxygen dissociation curve of the radular myoglobin is sigmoid, it lies far to the left of the corresponding curve for the vascular hemocyanin; hence, the presence of heme-heme interactions in the myoglobin does not interfere with the functional oxygen transfer system (Manwell, 1958c). Interactions between oxygen-affine centers have evolved in all four major classes of respiratory pigments (hemoglobin, hemocyanin, chlorocruorin, and hemerythrin); Bohr effects are found in all these classes except hemerythrin (reviewed by Prosser *et al.*, 1950).[3]

There is reason to believe that heme-heme interactions and the Bohr effect are not necessarily specialized acquisitions restricted to respiratory pigments in an advanced state of evolution but are expressions of very basic properties found in many unrelated proteins. The frequently observed variation of enzyme kinetics as a function of pH often involves interaction between proton-affine centers on the protein moiety and the active center (Alberty, 1956). Heme-heme interaction, likewise, has its parallel in the interaction between centers having similar reactivities in proteins possessing two or more such sites per molecule. Such interactions occur in the binding of dyes and ions to some multivalent proteins (Klotz, 1954) and in the kinetics of some enzymes (Botts and Morales, 1953).

Finally, the ease with which certain reagents (various mercurials, formaldehyde, and glutathione) will remove the heme-heme interactions, partially restore those interactions, greatly increase the oxygen affinity, and/or modify the Bohr effect (Guthe, 1954; Riggs and Wolbach, 1956) implies that these properties are not invariant for a particular hemoglobin molecule. In addition, the differences in the oxygen equilibrium of some hemoglobins inside and outside the red blood cell (Root, Irving and Black, 1939; Manwell, unpublished data) indicate also that a considerable lability exists with regard to the properties of the oxygen equilibrium.

It seems reasonable to assume that the phylogenetic order of first appearances was: heme-containing respiratory enzymes, tissue hemoglobins, vascular hemoglobins. However, the present discussion indicates the difficulty of knowing (a) if a certain set of characteristics of the oxygen-hemoglobin equilibrium—*e.g.*, high oxygen affinity, no heme-heme interactions, and no Bohr effect—is basically primitive, and (b) if any particular component of the cytochrome system or any other heme-containing enzyme is evolutionally the forerunner of hemoglobin.

SUMMARY

1. Oxygen equilibria of hagfish hemoglobin inside and outside the red blood cell have been obtained under a variety of conditions. The oxygen affinity of the hemoglobin in the erythrocyte suspensions is high (p_{50} = 3–4 mm. Hg at 18°), although it is even higher in hemoglobin solutions (p_{50} = 1.8 mm. Hg at 18° C.). There is no interaction between hemes ($n = 1.00$) and virtually no Bohr effect. The effect of temperature on the oxygen equilibrium of hagfish hemoglobin is

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[3] Absence of the Bohr effect has been confirmed for various sipunculid coelomic hemerythrins (Manwell, 1958a, and unpublished studies on *Dendrostomum zostericolum* and *Siphonosoma ingens*); however, the coelomic hemerythrin of the brachiopod *Lingula*, a form that is morphologically essentially unchanged since the Cambrian period, has a Bohr effect that is two-thirds the magnitude of that observed for human adult hemoglobin (Manwell, 1958, unpublished experiments)!

similar to that observed in recent experiments on other hemoglobins ($\Delta H^\circ = -9.3$ kcal. for hagfish hemoglobin).

2. Several aspects of Wald's (1952; see, also, Wald and Allen, 1957) theories on the evolution and function of hemoglobin are criticized in view of these data on hagfish hemoglobin and on the basis of information in the literature. It is concluded that: (1) At present there is no reason to favor cytochrome oxidase as *the* phylogenetic precursor of hemoglobin. (2) Many invertebrate hemoglobins function in oxygen transport. (3) If the internal oxygen tensions are sufficiently low, a respiratory pigment participating in oxygen transport does not need to possess a low oxygen affinity, a sigmoid oxygen dissociation curve, and a marked Bohr effect. (4) It is impossible to say if a particular set of properties of the oxygen equilibrium is basically "primitive." (5) Physiological conclusions on hemoglobin should be made upon studies of the pigment *in the natural condition—i.e.*, myoglobin in the muscle, or intracellular vascular hemoglobin in the erythrocyte.

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TOXIC EFFECTS OF NORMAL SERA AND HOMOLOGOUS ANTISERA ON THE CHICK EMBRYO[1]

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The possibility of identifying embryonic antigens of unique function, of localizing their sites of origin and action, and of manipulating them experimentally in order to analyze their developmental significance by the use of specific toxic sera at lethal or sub-lethal doses was advanced by Nace (1955). By modifying the normal function of an antigen with sub-lethal doses of a toxic antiserum, a specific anomaly may be produced, affording a key to the localization and the time and nature of the action of the antigen. Similar arguments have been advanced by those who have sought to block the growth of tumors with specific antisera (reviewed by Ross, 1957; Wissler and Flax, 1957). However, before this approach can be employed critically in studying the synthesis of specific antigens and their role in development, the following questions must be considered: (1) Are the proteins and other macromolecules of the embryo antigenic? Or does the embryo contain a population of molecules capable of reacting with antibody produced against adult antigens but incapable of eliciting antibody production? The distinction must be made between the occurrence in embryos of combining groups identical with those of adult antigens and the occurrence of embryonic antigens (Ebert, 1958a). (2) What are the effects of antisera on the embryo? Does the reaction between antigen and antibody, *in vivo*, result in measurable modifications of, or interference with, biological function? As a general rule, tissue-specific molecules exhibit species-specificity to some degree, making analysis by immunochemical techniques possible; the principal advantage of these methods is their exquisite sensitivity, which makes possible the analysis of the rate of synthesis and accumulation and site of localization of proteins or other macromolecules present in embryos in trace amounts. The principal difficulty, one which is often not appreciated, is that antigenic specificity depends upon relatively small determinant groups rather than on the complete structure of the molecule, and that the molecule may contain more than one kind of determinant group. Little is known of the kind, number, and size of determinant groups of natural proteins. The antigenically active groups and physiologically active groups of a molecule may not be identical. Similarity in immunochemical properties of natural molecules of diverse origin may result from (1) identity of one or more antigenic determinant groups, (2) a degree of structural similarity, or (3) the presence of one as an undetected trace contaminant in the other, for, inasmuch as traces of antigen may evoke large amounts of antibody, such contaminants tend to be revealed (Ebert, 1958b).

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[1] This investigation was supported in part by grants to Professor James D. Ebert from the National Science Foundation, the American Heart Association, the National Heart Institute of the National Institutes of Health of the United States Public Health Service (grant No. H-1709), and the Indiana University Foundation Research Division.

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The antigenicity of embryonic tissues has been demonstrated in several species, for example in amphibians (Cooper, 1948, 1950; Flickinger and Nace, 1952; Clayton, 1953) and in the sea urchin (Perlmann, 1954; Perlmann and Perlmann, 1957). Much of the earlier literature has been reviewed by Tyler (1955, 1957).

The ability of the chick embryo to react with antisera against tissue-specific components of adult chickens was shown by Burke *et al.* (1944), Nace and Schechtman (1948), Ebert (1950, 1951), and Ebert *et al.* (1955). For example, specific effects of antibodies against adult antigens on the development of chick embryos were reported by Ebert (1950). Saline extracts of heart, brain, and spleen from adult chickens were used as antigens. Rabbit antiserum against chicken brain affected chiefly nervous tissue, and anti-heart and anti-spleen sera affected primarily mesodermal elements in early chick blastoderms cultivated *in vitro*. Moreover, comparing the effects of anti-heart and anti-spleen preparations, it was clear that the former antisera affected the development of the heart, whereas the latter did not. A striking extension of this approach is found in the report by Langman *et al.* (1957) who have demonstrated specific effects of antisera developed in rabbits against antigens of chick lens, purified alpha crystallin, and myosin. Anti-lens and anti-alpha crystallin sera prevented the formation of the lens from ectoderm-optic cup combinations *in vitro*, whereas anti-myosin sera permitted normal lens development but inhibited mesenchyme formation. These studies clearly demonstrate the existence of reactive or combining groups in the embryo capable of reacting with antibodies to adult tissue antigens. However, the antigenicity of the chick embryo, *i.e.*, its ability to elicit the production of precipitating antibodies in the rabbit, cannot be inferred from these studies.

After complete absorption with adult laying hen serum, antisera against the serum of the 10-day chick embryo showed positive precipitating activity with the homologous antigens (Schechtman *et al.*, 1954). Moreover, Levi and Schechtman (1954) concluded from similar studies that the 12-day embryo contained distinct embryonic red blood cell antigens. Nettleship (1953) injected 1-day, 2-day, and 6-day chick embryos (p. 325), "emulsified in normal saline by syringe suction and expulsion or grinding in a mortar without abrasive," into hamsters. The hamster anti-chick sera were dropped or injected into or near the "embryo site" of unincubated eggs. The titers of the antisera used were not determined, nor were the antisera against the various stages tested with the homologous embryos. The hamster anti-chick embryo serum (p. 326) "placed in proximity to the preincubated chick embryo stops the development of these embryos at a time which corresponds to the time the embryo antigen was obtained." The results were interpreted as pointing strongly (p. 327) "to the development of qualitatively different protein complexes in the embryo concurrent with the embryo's growth." These results are consistent with the studies of Cooper (1950), Spar (1953), Nace (1953), and Flickinger and Nace (1952) showing changes in the antigenic constitution of the developing embryo. However, critical absorption techniques, such as those of Cooper (1950) and Spar (1953) in which antisera to later stages were absorbed with antigens of the earlier stage, thus separating those antibodies peculiar to the later stages, were not reported. Tyler (1957) remarked (p. 356) that "For an unabsorbed antiserum of this type to produce a highly specific effect does seem surprising, and one wonders whether or not this might be due to fortuitous variation in the antibody content of the various antisera."

The present experiments were undertaken with two-fold objectives: (1) To establish whether or not the chick embryo contains specific antigens (as opposed to combining groups capable of reacting with antibodies to adult tissues) by injecting whole chick embryos or embryo extracts into rabbits, and (2) to examine the effects of the antisera thus produced on the development of the homologous embryo. However, because fresh rabbit serum was found to be highly toxic to the chick, it became necessary first to study the known heat-labile and heat-stable factors in fresh rabbit serum in order to devise means of reducing or removing false reactions, after which the action of antisera could be explored.

Materials and Methods

Preparation of antigens

The 72-hour chick embryo (stages 16 to 18, Hamburger and Hamilton, 1951) was used for the preparation of antigens because of the significant number of well defined histogenetic and morphogenetic processes which occur during this period, *e.g.*, morphogenesis and the growth of the limb buds, the deposition of the pigment in the eyes. Another factor in selecting the 72-hour embryo was its larger size and higher content of protein nitrogen as compared with that of the 48-hour embryo. Even in using the 72-hour embryo, a substantial number of embryos was required for the preparation of the antigens. For example, it required approximately twenty 72-hour embryos to furnish material for one injection into a single rabbit.

The embryos were cut from the yolk and transferred immediately into a dish containing ice cold 0.15 *M* NaCl. The adhering yolk was trimmed off with sharpened steel needles, and all membranes were removed with sharpened jewelers forceps. The embryos were rinsed several times in ice cold saline and stored in the freezer (−20° C.) until used.

Saline extracts of the 72-hour embryo were prepared by permitting the frozen embryos to thaw at room temperature, after which they were homogenized with ice cold saline in a chilled Ten Broeck grinder. Approximately 1 ml. of saline was added for each 5 embryos. The cloudy suspension was refrigerated for 10 to 12 hours, after which it was centrifuged at 3000 RPM (1200 RCF) for 30 minutes at 0 to 4° C. The protein nitrogen content of the resultant translucent extract was approximately 0.2 mgN/ml., as determined by semi-micro Kjeldahl method.

Preparation of antibodies

In preliminary experiments, 6 white rabbits weighing 2 to 3 kilograms were injected intravenously with the saline extract and intraperitoneally with homogenized 72-hour chick embryos. Although injections and booster shots were given repeatedly, antisera with workable titers were not obtained. In subsequent experiments, 10 rabbits were injected with antigens in adjuvant; 72-hour chick embryos and equal amounts of Falba, paraffin oil and heat-killed tubercle bacilli were ground together in a mortar without abrasive and emulsified by syringe suction and expulsion. Each rabbit received approximately 10 to 12 embryos (at least 10 mg. of protein) in each of three injections administered subcutaneously in the region of the neck, one week apart. The rabbits were bled from the marginal vein of the ear one month after the first injection and one week after an intravenous booster shot of 10 to 20 embryos homogenized in a small amount of saline. This method yielded antibodies with titers of 1 : 32 to 1 : 128. By standards conventionally employed for antibodies against purified antigens, these titers are low. As will be made clear, however, they proved to be valuable tools.

Tests of antibody content

The presence of antibodies was detected by the use of interfacial "ring" tests in which 0.1 ml. of the test serum was overlaid with 0.1 ml. of the serially diluted chick embryo extract. The tests were performed in 6 × 50 mm. culture tubes and test materials were delivered with measuring pipettes ground and fitted with hypodermic needles at the tip. After the appearance of the rings was noted, the tubes were mixed and placed in the refrigerator overnight. The next morning the tubes were tapped gently and the presence of precipitate was detected as a thin white spiral rising from the bottom of the tube.

The titer of the antiserum used was determined by precipitin tests in which 0.1 ml. of the embryo extract was added to 1 ml. of the serially diluted antiserum. The tubes were then incubated in a water bath at 37° C. for 30 minutes. The tubes were read after incubation and again after being refrigerated overnight.

Operative procedures

A technique for the study of the effects of antisera on the chick embryo was described by Witebsky and Neter (1935), who added serum drop by drop to the embryo. A modification of this technique was adopted in this study. New Hampshire eggs obtained from a local hatchery were incubated for 72 hours at 37.5 to 38° C. Preparatory to operation, the egg was swabbed with 70% alcohol. Then a square window 1 cm. × 1 cm. was cut in the shell with a sharpened hack saw blade. The cut piece of shell was removed, and the shell membrane was cut off. After the embryo was in position immediately under the window, a small hole was made in the vitelline membrane just anterior to the heart, after which 0.05 to 0.1 ml. of the test serum was inserted. The material, especially if colored and dense, could be seen to envelop the embryo and remain in position for several hours. The window was sealed with cellophane tape, and the egg was returned to the incubator.

All test materials were sterilized by autoclaving or by Seitz filtration. After the latter procedure the titer of each serum was checked because of the report (Dilks and Wolfe, 1949) that significant decreases in titer result from Seitz filtration. In the present study, decreases in titer were minimized by filtering large volumes of undiluted serum.

More than 50 experiments were conducted. Each experiment consisted of at least 25 to 35 embryos, including sham-operated or saline controls, and normal or absorbed serum controls. Each embryo was numbered; the time of operation, the stage of the embryo, and the amount of the test substance administered recorded. All observations were recorded following examination of the specimens under a binocular dissecting microscope, after which the embryos were removed and dissected or fixed in calcium formol for further histological studies.

In the interest of objectivity, frequently the assistance of a second person was enlisted to code the randomly numbered treated eggs and to record the observations made by the experimenter.

The Effects of Normal Rabbit Serum on the 72-Hour Chick Embryo

In preliminary experiments, it was found that sera from both uninjected and injected rabbits were toxic to the embryo. Within a few minutes after the application of fresh rabbit serum the blastoderm begins to shrivel, the embryo gradually sinks and the heart stops beating. The toxicity of fresh rabbit serum has been encountered by others. Witebsky and Neter (1935) described its effects on the chick embryo. Bernheimer and Harrison (1940) observed the ability of normal rabbit serum to immobilize *Paramecium*. Green (1946) observed that normal rabbit serum interfered with the rapid growth of cancer cells, and Imagawa *et al.* (1954) observed that normal rabbit serum inhibited the proliferative capacity of mouse mammary cancer cells. A spermicidal factor in fresh human, bovine, rabbit, and rat sera was reported by Chang (1947). Nace has described normal rabbit sera which were toxic to the *Rana pipiens* embryo (1955; see also Nace and Inoue, 1957).

Table I

The effect of heating on the toxicity of fresh normal rabbit serum (NRS)

Serum	No. embryos treated	Effects on 72-hour chick embryos		
		Normal	Abnormal	Dead
Unheated NRS	117	3	3	111
NRS heated at 37° C. 30 minutes	8	0	0	8
NRS heated at 42° C. 30 minutes	8	1	0	7
NRS heated at 50° C. 30 minutes	10	5	0	5
NRS heated at 56° C. 30 minutes	17	17	0	0

Witebsky and Neter (1935) reported that the toxic effects of fresh rabbit serum were removed by heating at 56° C. for 30 minutes, an observation confirmed in the present study. Partial inactivation was obtained by heating at 50° C. for 30 minutes, but below 50° C. inactivation did not occur (Table I). What is the nature of the heat-labile substance? Is it *complement* which is defined in part on the basis of its destruction by heating at 56° C. for 30 minutes? Is it *properdin*, the heat-labile substance recently found in normal serum of a number of animals (Pillemer *et al.*, 1954), or is it another heat-labile substance as yet undescribed? Other questions may be asked, among them: does the heat-labile factor act independently, or does it require the presence of heat-stable and/or other heat-labile factors for its action? What is the mechanism of its action?

Table II

The toxicity of heated normal rabbit serum (HNRS) coupled with unheated chicken serum (CS) or unheated guinea pig serum (GPS)

Serum	No. embryos treated	Effects on embryos		
		Normal	Abnormal	Dead
HNRS	43	43	0	0
CS	29	26	0	3
HNRS + CS (10 to 50%)	11	11	0	0
GPS	40	38	2	0
HNRS + GPS (10 to 50%)	27	26	1	0

The role of complement in the toxicity of fresh rabbit serum was examined first. The sufficiency of complement was tested by adding complement in the form of fresh guinea pig serum or fresh chicken serum to heated normal rabbit serum. The toxicity which was characteristic of fresh rabbit serum was not restored to heated rabbit serum by the addition of either fresh chicken serum pooled from 4 to 6 chickens, or fresh guinea pig serum pooled from 4 to 6 guinea pigs (Table II).

Next, complement or components of complement were removed from rabbit serum by absorbing unheated fresh rabbit serum with a nonspecific precipitate which had been prepared by combining beef serum albumin (BSA) with heated homologous rabbit antiserum (anti-BSA). The precipitate was washed three times with cold saline, after which 10 ml. of fresh normal rabbit serum were added to 0.5 ml. of packed precipitate. The mixture was refrigerated (0 to 4° C.) for 8 to 12 hours with frequent stirring. The mixture was centrifuged; next the supernatant was poured into another tube containing 0.5 ml. of packed beef serum albumin precipitate. After three absorptions, the rabbit serum was unable to lyse chicken red blood cells. The fresh rabbit serum absorbed in this manner was still toxic to the 72-hour chick embryo. The toxicity was lost only after heating for 30 minutes at 56° C. (Table III). These experiments clearly demonstrate that the heat-labile substance, complement, which can be absorbed by a nonspecific precipitate, is neither sufficient nor necessary for the toxic action of fresh rabbit serum.

Table III

The toxicity of normal rabbit serum (NRS) absorbed in the cold with beef serum albumin (BSA) precipitate and chicken red blood cells (RBC)

Serum	Absorbed with	No. embryos treated	Effects on embryos		
			Normal	Abnormal	Dead
NRS	BSA	70	37	5	28
NRS	BSA then heated	33	32	0	1
NRS	RBC	36	6	3	27
NRS	RBC then heated	30	28	2	0
NRS	BSA and RBC	16	6	4	6
NRS	BSA and RBC then heated	10	10	0	0

To determine whether or not heat-labile factors were capable of acting independently, heat-stable substances found in normal rabbit serum were removed by absorption. Several substances can be used for absorption purposes, among them, chicken red blood cells which were selected because they contain Forssman antigens (Boyd, 1956). In this manner, Forssman antibodies, as well as other substances absorbable by chicken red blood cells, can be removed. To minimize the destruction of heat-labile substances during the absorption process, the procedure was conducted in the cold (0 to 4° C.). Red blood cells were obtained from the pooled blood of 4 to 6 adult New Hampshire chickens. Approximately 2 ml. of packed red blood cells were used in the absorption of each 10 ml. of serum. The red blood cells and serum were thoroughly mixed by frequent stirring. After 8 to 12 hours, the cells were removed by centrifugation at 1200 RCF for 30 minutes. The serum was poured into another tube containing 2 ml. of packed red blood cells. The process was repeated until no further agglutination of red blood cells was observed under the microscope. The cold-absorbed rabbit serum was still highly toxic to the 72-hour chick embryo.

Since lysis frequently occurred during the long course of absorption in the cold, it was necessary to inactivate complement by removing cations before absorbing with red blood cells either by filtering the serum through a column of cation exchange resin (IRC-50, Rohm and Haas Company, Philadelphia, Pa.), as described by Levine *et al.* (1953), or by adding Versene (sodium ethylene diamine tetraacetate) to the serum. The latter method was found to be more successful. Upon completion of absorption, calcium and magnesium ions were reconstituted to a final concentration of 0.00015 *M* and 0.0005 *M*, respectively (Mayer and Levine, 1954).

The cold-absorbed serum which contained Versene and an insufficient amount of calcium and magnesium ions was found to be toxic to the 72-hour chick embryo. The picture of toxicity, however, differed from that produced by fresh rabbit serum. Upon the injection of absorbed serum containing Versene (a 6 millimolar solution of Versene in serum), the embryo dies within a few minutes. The heart is engorged with blood and becomes bright red in appearance. However, the puckering of the blastoderm and the sinking of the embryo, which is characteristic of the effect of fresh rabbit serum, is not observed. The toxic effects of Versene are observed only when insufficient calcium and magnesium ions are present.

To determine whether or not complement was still present in the cold-absorbed rabbit serum, its ability to lyse chicken red blood cells was tested. The cold-absorbed serum was unable to lyse chicken red blood cells. However, when a sufficient amount of heated but unabsorbed rabbit serum was added (1 : 1), lysis occurred readily. Thus, complement, which is dependent on the presence of heat-stable substances, was not destroyed in the process of absorption in the cold. Again, the toxic effects of cold-absorbed rabbit serum can be removed by heating (Table III). This result demonstrates the presence of a toxic heat-labile substance in fresh rabbit serum, a substance not absorbed by chicken red blood cells.

Absorption of fresh rabbit serum with beef serum albumin precipitates followed by chicken red blood cells in the cold also failed to remove the toxicity of the serum (Table III). It thus may be concluded that complement is neither sufficient nor necessary for the toxic action of fresh normal rabbit serum which is evoked in the absence of substances absorbable with chicken red blood cells. Moreover, since

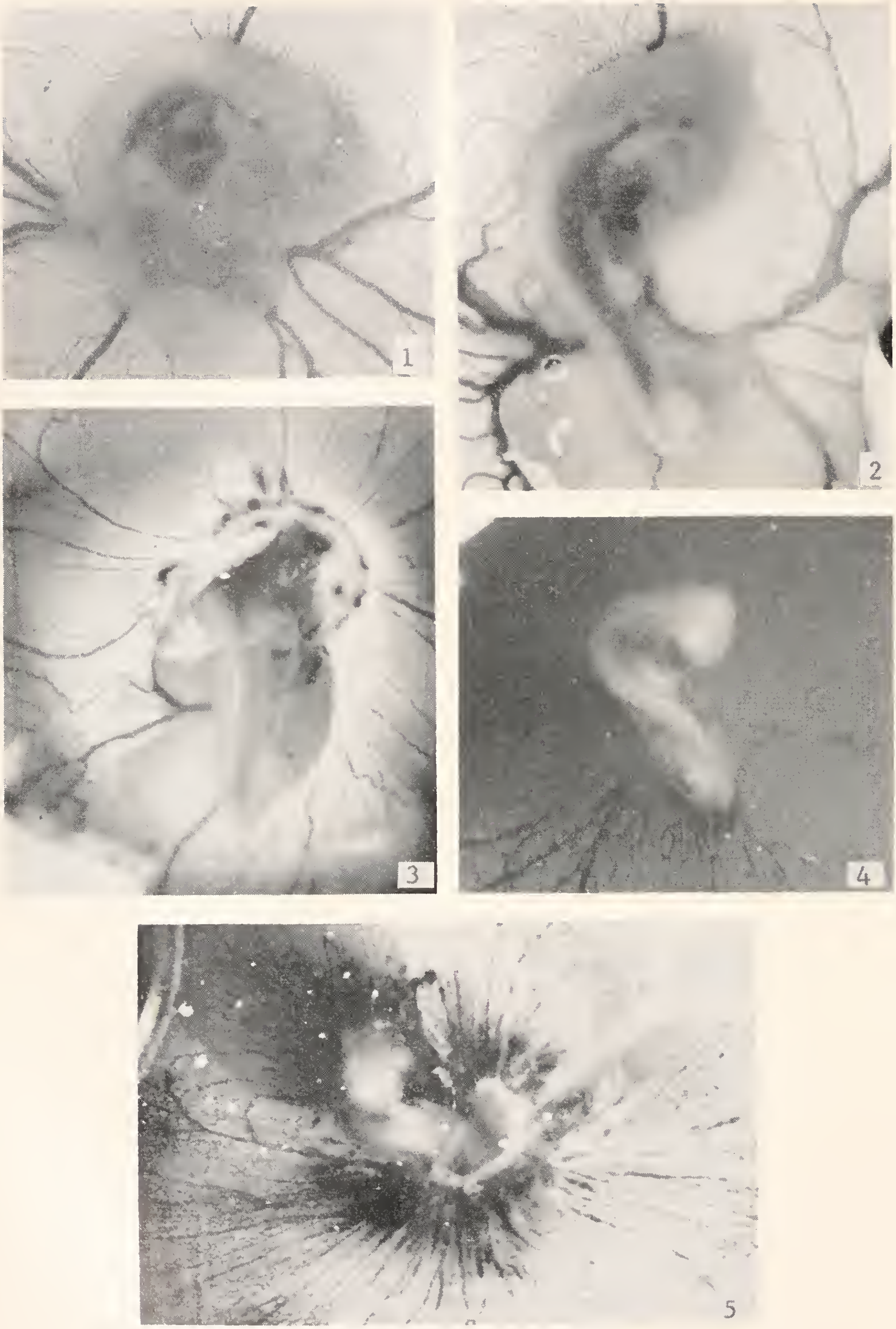

Plate I

Photographs were taken *in ovo* through the cut window 20–24 hours after treatment.

the action of properdin requires both complement and magnesium ions (Pillemer *et al.*, 1954), it is suggested that this heat-labile substance is not properdin.

The Effects of Antisera Against the 72-Hour Chick Embryo on the Homologous Embryo

It is clear from the foregoing experiments that to remove nonspecific toxic factors, normal rabbit sera and antisera must be heated for 30 minutes at 56° C. When heated rabbit antiserum against the 72-hour chick embryo was placed on the embryo, the immediate puckering of the blastoderm, together with its sinking, which was characteristic of fresh rabbit serum, was not observed. However, a number of the embryos died after 6 to 8 hours; in some cases the embryos did not show any visible effects until 15 to 18 hours after the operation, at which time slight abnormalities were detected. Usually no further changes appeared in the surviving embryos after 18 to 20 hours. Occasionally, some of the embryos with slight visible abnormalities appeared to recover completely. The toxic effects of the sera on the chick embryo arbitrarily are divided into five different groups (Table IV; Plate I).

Table IV

The effects of heated rabbit antiserum against the 72-hour chick embryo (HA72) coupled with guinea pig serum (GPS)

Serum	No. embryos treated	Graded effects on 72-hour chick embryos				
		1	2	3	4	5
HNRS	60	59	1	0	0	0
A72	51	1	0	0	0	50
HA72	57	28	7	6	16	0
HNRS + GPS	27	25	1	1	0	0
HA72 + GPS	69	19	3	8	6	33

Plate I

Group 1. The embryos appear essentially normal with good color, as compared with unoperated embryos of the same stage (Fig. 1).

Group 2. The embryos appear essentially normal in stage and color but show slight morphological abnormalities, *e.g.*, the trunk may be turned ventrad, instead of to the left in embryos in stage 22. These abnormalities may be detected 15 to 18 hours after the operation (Fig. 2).

Figure 1. A group 1 embryo which is alive and appears normal (6 ×).

Figure 2. A group 2 embryo with accumulation of blood in trunk region (6 ×).

Fig. 3. A group 3 embryo which is alive with its head beneath the puckered portion of the blastoderm (6 ×).

Figure 4. A group 4 embryo which is dead. The embryo lies on top of the blastoderm which is smooth in appearance (6 ×).

Figure 5. A group 5 embryo which is dead and partially hidden by the puckered blastoderm (6 ×).

Group 3. The embryos are alive but show distinct abnormalities, *e.g.*, the trunk may be turned to the left or even doubled back upon itself. The embryos are usually pale in color. These abnormalities may be detected 10 to 12 hours after operation (Fig. 3).

Group 4. The embryos are dead and appear quite small and shrunken; blood vessels are not distinct. These embryos usually die 5 to 8 hours after the operation (Fig. 4).

Group 5. The embryos are dead. The blastoderm appears puckered or pursed. The blastoderm may be seen to begin to shrivel 3 to 5 minutes after the operation. The red blood cells may be seen to clump in the blood vessels in a few minutes and then cease to flow in the smaller vessels. The heart may stop beating as soon as 5 minutes after the operation (Fig. 5).

This classification of the extent of the toxic action on the embryo does not imply the expression of basically different mechanisms or functions in each of the five groups, nor does it indicate distinct and separate stages or steps of a single mechanism or function. The embryos earlier described as "normal" fall into either group 1 or 2. Embryos described as "abnormal" are similar to those in group 3, whereas embryos described as "dead" fall into either group 4 or 5. The low sensitivity of the system, probably owing in part to the low titer of the antisera employed (1 : 32 to 1 : 128), as well as to the heating of the antisera, increased the possibility of introducing false negative reactions. Methods were sought, therefore, to increase the sensitivity of the system. From the foregoing discussion, it is apparent that methods to achieve this end are available; *viz.*, the expedient of adding back those substances which are destroyed by heating, such as complement and properdin, but which do not contribute to the toxicity of normal rabbit serum.

The Effects of Guinea Pig and Rat Serum on the Activity of Rabbit Antiserum Against the 72-Hour Chick Embryo

The role of complement *in vivo* is not fully understood. It is needed in addition to antibody for bactericidal and hemolytic reactions of immune sera, as well as for other toxic effects (Boyd, 1956). Witebsky and Neter (1935) found that adding fresh guinea pig serum to heated rabbit antiserum against sheep red blood cells restored the toxic activity of the antiserum but not that of normal rabbit serum. Imagawa *et al.* (1954) showed that antisera produced in guinea pigs against mouse cancer cells when heated lost the ability to inactivate mammary cancer cells but that this activity could be restored by the addition of fresh guinea pig complement. Therefore, because previous experiments showed that complement was neither sufficient nor necessary for the toxicity of fresh rabbit serum, in an attempt to increase the effectiveness of the heated antiserum, complement was returned to the heated rabbit antiserum in the form of fresh guinea pig serum.

Fresh unheated guinea pig serum, obtained from the pooled blood of 4 to 6 guinea pigs, had no visible effects on the 72-hour chick embryo. Fresh guinea pig serum, when mixed with heated normal rabbit serum in varying proportions, also showed no visible effects. However, a mixture of guinea pig serum and heated antiserum against the 72-hour chick embryo was quite toxic to the 72-hour chick embryo, resulting in embryos in the group 5 condition (Table IV).

The effects of the addition of a second heat-labile substance, properdin, were examined next. Pillemer *et al.* (1954) found properdin in high concentration in the rat (25–50 units properdin/ml. serum), in intermediate concentration in the rabbit (4–8 units properdin/ml. serum), and in low concentration in guinea pig serum (1–2 units properdin/ml. serum). Therefore, rat serum was chosen as the source of properdin. Fresh rat serum was obtained from the pooled blood obtained by cardiac punctures from 4 to 6 large white rats. Fresh rat serum alone was highly toxic to the 72-hour chick embryo, producing the striking vascular phenomena described previously at all concentrations above 4%. Heating for 30 minutes at 56° C. removed all observable toxic effects. Preliminary experiments in which the embryos were examined 5 minutes to 4 hours after treatment showed that 4% fresh rat serum mixed with heated normal rabbit serum was extremely toxic to the 72-hour chick embryo.

In the case of rat serum, the titer of complement is low but the concentration of properdin was shown to be high, whereas, in the case of the guinea pig serum, the titer of complement is high but the concentration of properdin is low; therefore a study of the combination of rat and guinea pig serum, together with rabbit serum, was conducted. A mixture of fresh rat serum and guinea pig serum, at a dilution in which neither was capable of eliciting toxic effects alone, was quite toxic to the chick embryo. The toxicity of this mixture was also increased when heated normal rabbit serum was added to this mixture. The toxicity was lessened when the concentration of the heated normal rabbit serum was reduced by dilution with saline (1:2 to 1:4). These experiments suggest the possible interaction of heat-labile substances in guinea pig and rat sera with heat-stable substances in guinea pig, rat, and normal rabbit serum. Thus, the following absorption studies were conducted to remove nonspecific heat-stable substances.

Absorption Studies

Forssman antigen is reported to be present in the tissues of the chick embryo from the beginning of its development. Heated rabbit antiserum against sheep red blood cells mixed with guinea pig serum evoked the characteristic vascular phenomenon in the early chick embryo, whereas heated normal rabbit serum mixed with guinea pig serum would not (Witebsky and Neter, 1935). Therefore, it appeared imperative that Forssman type antibodies formed as a result of the injection of chick embryos into the rabbit be removed by absorption with chicken red blood cells (RBC). This procedure increased the specificity of the reaction but, owing to the concomitant dilution, decreased the sensitivity. When heated antiserum against 72-hour chick embryos was absorbed with chicken red blood cells at 37° C., the proportion of embryos showing the group 4 condition was decreased (Table V).

Adding fresh guinea pig serum increased the toxicity of the heated and absorbed antiserum. Several embryos in the group 5 condition were observed. Absorption of the fresh guinea pig serum with chicken red blood cells in the cold in the presence of Versene decreased the action of the heated and absorbed antiserum and absorbed guinea pig serum combination (Table V). This result may have been due to some inactivation of complement during the process of absorption.

As shown previously, fresh rat serum was extremely toxic to the 72-hour chick embryo at concentrations above 4%. After absorption with chicken red blood cells in the presence of Versene in the cold (0 to 4° C.), the rat serum was no longer toxic to the embryo at concentrations below 10%. A mixture of 6% absorbed rat serum and 94% heated and absorbed rabbit antiserum was without effect on the embryo, as was a mixture of 6% rat serum and 94% heated and absorbed rabbit antiserum (Table VI). This is in strong contrast to the boosting effect of the addition of guinea pig serum to the heated rabbit antiserum. However, this finding is not unexpected, because properdin acts only in conjunction with complement and magnesium ions (Pillemer *et al.*, 1954) and the concentration of complement in rat serum is low (Hegedüs and Greiner, 1938).

A mixture of guinea pig serum and rat serum at a dilution in which neither could elicit toxic effects was shown to be extremely toxic to the 72-hour chick embryo. After absorption of the rat serum in the cold with RBC following filtration through a cation exchange (IRC-50) column, the toxic activity of the rat and guinea pig serum mixture was decreased.

TABLE V

The effects of absorption on the toxicity of heated rabbit antiserum against 72-hour chick embryos (HA72) and unheated guinea pig serum (GPS) combinations

Serum	Absorbed with	Combined with	No. embryos treated	Graded effects on embryos				
				1	2	3	4	5
HNRS	RBC	None	52	40	9	3	0	0
HA72	None	None	57	28	7	6	16	0
HA72	RBC	None	45	30	2	6	7	0
HNRS	RBC	GPS	19	18	1	0	0	0
HA72	RBC	GPS	63	5	7	8	3	40
HA72	RBC	GPS, heated	23	10	6	2	5	0
GPS	RBC	None	7	7	0	0	0	0
HNRS	RBC	Absorbed GPS	14	13	1	0	0	0
HA72	RBC	Absorbed GPS	17	3	3	1	4	6

The toxicity of a mixture of 6% fresh rat serum, 10% guinea pig serum, and 84% heated normal rabbit serum mixture was also reduced or removed altogether by the absorption of the guinea pig serum and rat serum at 0 to 4° C. with chicken red blood cells in the presence of Versene, and by the absorption of the heated normal rabbit serum at 37° C. with chicken red blood cells. On the other hand, a similar mixture of 10% absorbed guinea pig serum, 6% absorbed rat serum, with 84% heated and absorbed rabbit antiserum was toxic to the embryo. Immediate vascular effects were observed, followed by the cessation of heart contractions within 30 minutes (Table VI). The proportion of embryos exhibiting toxic effects was greater in this rabbit antiserum mixture containing both guinea pig serum and rat serum than that in rabbit antiserum mixtures containing either guinea pig serum or rat serum alone. Hence, a method is available to increase the effectiveness of antisera. Since rat serum enhanced the effect of the antiserum only in the presence of complement, it is suggested that a factor or factors analogous to properdin may be involved. Need for further experiments using purified properdin is indicated.

Properdin can participate in such diverse activities as the destruction of bacteria, the neutralization of viruses, and the lysis of certain red blood cells (Pillemer *et al.*, 1955). Although the presently reported experiments suggest the interaction of "the properdin system" with this specific antibody in the serum (A72), it is possible that the rat serum acts by supplementing the components of complement which are low in both rabbit and guinea pig sera, *e.g.*, the C'1 component (Hegedüs and Greiner, 1938). The application of quantitative techniques for handling complement and the use of purified components of complement, C'1, C'2, C'3, and C'4, may elucidate this aspect of the problem.

The absorption of the antiserum against 72-hour chick embryos with the homologous antigen removed most of the toxic effects of the antiserum on the 72-hour chick embryo. The antiserum was first heated and absorbed with chicken red blood cells in the manner described previously, and then mixed with a slight excess of minced and homogenized 72-hour chick embryos. The suspension was placed in the water bath for two to three hours at 37.5° C. A dense white precipitate was usually observed after 15 to 30 minutes. The tube was placed in the refrigerator at 0 to 4° C. for 12 hours and later centrifuged at 1200 RCF for 30 minutes. The supernatant was poured off into another tube containing a slight excess of homogenized 72-hour chick embryos. A smaller amount of precipitate was observed after the second absorption. The process was repeated until a negative interfacial "ring" test was obtained.

TABLE VI

The effects of the addition of absorbed rat serum (RAT-RBC) and absorbed guinea pig serum (GPS-RBC) to heated and absorbed antiserum against the 72-hour chick embryo (HA72-RBC)

Serum	No. embryos treated	Graded effects on embryos				
		1	2	3	4	5
RAT-RBC (100%)	10	0	1	1	2	6
RAT-RBC (6 to 10%)	8	8	0	0	0	0
HNRS-RBC (94%) + RAT-RBC (6%)	9	9	0	0	0	0
HNRS-RBC (84%) + GPS-RBC (10%) + RAT-RBC (6%)	16	14	2	0	0	0
HA72-RBC (100%)	45	30	2	6	7	0
HA72-RBC (94%) + RAT-RBC (6%)	18	9	8	0	1	0
HA72-RBC (84%) + GPS-RBC (10%) + RAT-RBC (6%)	32	5	2	2	5	18

When guinea pig and rat sera were added to this heated antiserum which had been absorbed with both chicken red blood cells and 72-hour chick embryos, the toxic effects of the antiserum were not found to be completely removed (Table VII). The failure of the absorption of the antiserum by the homologous antigen is surprising but not without precedent. Ebert (1950) reported the failure of absorption by homologous antigen to remove the striking lethal and growth inhibitory powers of anti-organ sera. This non-absorption of one fraction of the antiserum was attributed to individual differences in the organ antigens used in injections

Table VII

The toxicity of heated antiserum against the 72-hour chick embryo (HA72) absorbed with the homologous antigen, singly, and in combination with absorbed guinea pig serum (GPS) and absorbed rat serum (RAT)

Serum	Absorbed with	No. embryos treated	Graded effects on embryos				
			1	2	3	4	5
HA72	RBC	11	8	1	0	2	0
HA72	RBC, 72-hour chick embryos	23	21	2	0	0	0
HA72 (90%) + GPS (10%)	RBC	57	3	5	7	3	39
HA72 (90%) + GPS (10%)	RBC, 72-hour embryos	34	21	6	2	4	1
HA72 (94%) + RAT (6%)	RBC	23	14	8	0	1	0
HA72 (94%) + RAT (6%)	RBC, 72-hour embryos	12	8	0	1	3	0
HA72 (84%) + GPS (10%) + RAT (6%)	RBC	32	5	2	2	5	18
HA72 (84%) + GPS (10%) + RAT (6%)	RBC, 72-hour embryos	14	7	1	1	1	4

and absorptions. Although large numbers of embryos were used in both injections and absorptions, a long course of injections was given. Such treatment often results in antisera of reduced specificity. This result may be even more pronounced in animals receiving adjuvant. However, the injection of adjuvant with heterologous antigen was insufficient to evoke a nonspecific response in the rabbit. Beef serum albumin (BSA) combined with adjuvant was injected into three rabbits. Tests of heated anti-BSA, and heated anti-BSA absorbed with BSA were negative (Table VIII). The number of different kinds of antibodies may be so great as to be incompletely absorbed by the antigen, even though an excess of antigen was used in absorptions and negative interfacial "ring" tests were obtained after the final absorption. This is not to say, however, that antibodies with new and different specificities are formed. The *in vivo* system employed here may be so sensitive as to respond strongly to these weaker or less "avid" antibodies. That embryonic proteins may be unique in their behavior in precipitin reactions was reported by Schechtman (1952), who found an unusual result when antiserum against the plasma from the 10-day embryo was reacted with adult and 10-day

Table VIII

Effects of antiserum against beef serum albumin (ABSA) on the 72-hour chick embryo

Treatment of serum	No. embryos treated	Effects on embryos				
		1	2	3	4	5
ABSA unheated	3	0	0	0	0	3
ABSA heated	7	6	1	0	0	0
ABSA heated and absorbed with RBC + GPS	15	15	0	0	0	0
ABSA heated and absorbed with RBC and BSA + GPS	6	6	0	0	0	0

serum. He wrote (p. 95), "This antiserum forms higher (antigen-antibody precipitation) curves with the heterologous antigen, adult serum. The antiserum is obviously not lacking in antibody since it produces heavy precipitates with adult material." He concluded that the embryonic serum forms antigen-antibody complexes with inferior light-scattering properties or that it contains substances inhibitory to the precipitin reaction.

Discussion

The toxicity of fresh rabbit serum to the early chick embryo was destroyed by heating at 56° C. for 30 minutes. The above experiments show clearly that the toxic substance in fresh rabbit serum is not complement; nor is it dependent on complement for its activity. In view of the latter observation, it is also probably not properdin. The following questions remain to be answered: (1) What are the physicochemical properties of this toxic heat-labile substance? (2) Is is composed of one or many substances? Can substances, other than complement, be separated or isolated from this heat-labile fraction which would further enhance the action of heat-stable fractions, as was shown above for complement and properdin or properdin-like substances? (3) What is the mechanism of action of this heat-labile substance? Is it similar to that brought about by heat-stable fractions? It was observed that the toxic effects of fresh normal rabbit serum in general resembled those produced by the action of heated rabbit antiserum to the 72-hour chick embryo coupled with fresh guinea pig serum and rat serum. Witebsky and Neter (1935) also described similar toxic effects on the early chick embryo of heated rabbit anti-sheep red blood cell serum plus complement. Pomerat (1949) reported similar results with rabbit anti-chick spleen serum. However, although the final picture appears to be the same, the mechanisms involved may not be similar. The development and use of more specific antisera to embryonic antigens may reveal more definitive and specific morphological expressions than those elicited by toxic factors in fresh rabbit serum. The present study has demonstrated that the 72-hour chick embryo is antigenic, *i.e.*, capable of eliciting the production of precipitating antibodies.

The presently reported investigation also demonstrated the fact that complement and properdin or properdin-like substances can play an active role in the action of the antiserum *in vivo*. The demonstrated ability of complement and properdin or properdin-like substances to increase the magnitude of the action of the antiserum will permit the observation of the effects of weaker but perhaps more specific antisera which otherwise would go unnoticed. Thus, the manner in which antisera act to block development or modify the normal function of reactive groups in the embryo may be studied more readily. The use of purified properdin or related substances, together with the components of complement, C'1, C'2, C'3, and C'4, may contribute to our understanding of the mechanism of action of the toxic antiserum *in vivo*.

I wish to express my sincere appreciation to Professor James D. Ebert for his encouraging interest and valuable advice throughout the course of this investigation, and to Dr. Royal F. Ruth for his many helpful suggestions. I also wish to thank Dr. Joseph F. Albright and Mr. Lowell M. Duffey for expert technical aid.

SUMMARY

1. The specific objectives of the present investigation were at first two-fold: (1) to determine the antigenicity of the early chick embryo, and (2) to study the effects of homologous antisera on the chick embryo. However, because at the outset a profound toxic action of fresh normal rabbit serum was encountered, it became imperative to describe the toxic factor.

2. The toxic action of normal rabbit serum, characterized by the puckering of the blastoderm, the sinking of the embryo and its ultimate death, was removed by heating at 56° C. for 30 minutes. The toxic action was not restored by adding fresh guinea pig serum to heated rabbit serum. The toxicity was not removed by absorption in the cold with nonspecific antigen-antibody precipitates and/or chicken red blood cells. These results are interpreted as indicating that complement is neither necessary nor sufficient for the toxic action of fresh rabbit serum. The toxic heat-labile substance can also act independently of heat-stable substances which are removed by absorption with chicken red blood cells.

3. The antigenicity of the 72-hour chick embryo was demonstrated by its ability to elicit the production of precipitating antibodies in the rabbit. Heated rabbit antiserum against the 72-hour chick embryo evoked a weak but definite toxic response when placed on the homologous embryo.

4. In an attempt to decrease the probability of false negative reactions, methods were sought to increase the effectiveness of the antisera. Substances which may have been inactivated by heat were returned to the antiserum singly and in combination.

5. The toxic action of heated rabbit antiserum was partially enhanced by the addition of fresh guinea pig serum, rich in complement.

6. The toxic action of the heated rabbit antiserum was not increased by adding fresh rat serum, reported to contain large amounts of properdin, but was enhanced by a mixture of guinea pig serum and rat serum.

7. The results suggest the interaction of complement and properdin or a properdin-like factor in the action of the antiserum on the chick embryo.

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THE DYNAMICS OF A DIATOM BLOOM [1]

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Phytoplankton cells respond so rapidly to their environment that conventional methods of studying their populations fail to reveal many of the more subtle and more interesting aspects of their dynamic ecology. This is particularly true of surveys in which observations are made at intervals of weeks or months, where the very use of the term "the phytoplankton population," when carried over from one set of observations to the next, implies more knowledge than is available. It is also true of productivity measurements made over 24 hours or the daylight portion of a day, since Rodhe (personal communication), Doty and Oguri (1957), Yentsch and Ryther (1957) and others have shown that the plants vary in their composition and react differently to their environment at different times of the day. To study such phenomena it is obviously necessary to make intensive observations at very frequent intervals throughout one or more days on single, isolated populations.

This type of study presents obvious difficulties. The average natural population is sparse enough so that its properties can be measured only with rough accuracy, and the errors of such measurements may be larger than the changes in the organisms and their environment which are under investigation. Some insight into these problems may be had by studying cultures, but the difficulties of growing organisms under completely natural conditions need no elaboration here. A compromise may be reached, however, by working with a dense phytoplankton bloom. Here natural populations may be studied under their natural growing conditions and very rapid responses of the organisms to changes in their physical or chemical environment may be detected and measured.

The authors encountered such a diatom bloom in a small tidal creek on the south shore of Long Island, N. Y., in June, 1957. The following report will describe the studies of this bloom which were made over a 40-hour period including two days and one night.

Description of the Area

Senix Creek is approximately one mile long, tapering from a width of about 300 meters at its mouth to less than 10 meters at its upper end in the town of Center Moriches. Our observations were made about halfway up its length where the water depth is approximately one meter. Underlying this shallow body of water is a thick deposit of black, organic muck which discharges H_2S gas when disturbed.

There is no river or other obvious source of fresh water to Senix Creek except for local runoff. The latter was negligible in the early summer of 1957 due to abnormally low rainfall (none whatever during the month of June). This drought was undoubtedly an important contributory factor to the existence of the diatom bloom, since it helped to maintain the salinity at a moderately high level in the estuary and also reduced flushing action so that the latter did not greatly influence the population at the time and place of our observations.

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[1] Contribution No. 978 from the Woods Hole Oceanographic Institution. This study was made with the assistance of research grants from the National Science Foundation.

While many of the tributaries to Moriches Bay receive large quantities of pollutants from duck farms which line their shores (see Ryther, 1954), there is no such direct source of enrichment to Senix Creek. The origin of the nutrients which gave rise to the phytoplankton bloom in question was not investigated. They presumably resulted either from domestic pollution from Center Moriches and the residences located along the banks of the creek, or from an invasion of water from one of the other, heavily polluted estuaries via Moriches Bay (*i.e.*, Forge River located just ½ mile from Senix Creek at their respective mouths).

Methods and Procedure

The studies were initiated at sunrise on June 26, with the plan to make observations hourly during the day and less frequently at night for 24 hours. Unfortunately, the day was foggy and partially overcast, and it seemed doubtful that we would be able to observe phenomena associated with high incident light intensities. Consequently we continued the study for the daylight portion of a second day, when the sky was clear.

Incident radiation was measured at one-hour intervals throughout the day with a GE radiation meter. Light penetration was measured with a submarine photometer constructed from a Weston photronic cell. Water level, measured with an improvised tide gage, was also recorded hourly during the day, less frequently at night. At these same time intervals water samples were collected in a bottle equipped with a siphon which permitted the sample to enter through a tube running to the bottom of the bottle and the air to escape through a tube extending to the surface of the water. In this way samples for gas analysis were not contaminated by bubbles. As the bottle filled it was slowly lowered from the surface to the bottom, thereby obtaining an integrated sample of the one-meter water column. Immediately after collection, the water temperature was taken and an aliquot of the sample was siphoned into a 150-ml. glass-stoppered bottle and analyzed for dissolved oxygen using the Pomeroy-Kirschman-Alsterberg modification of the Winkler method (see APHA, 1955). The pH of the sample was measured with a Coleman pH meter, and a 100-ml. aliquot was withdrawn and millipore-filtered for subsequent pigment analysis using the method of Richards with Thompson (1952) as modified by Creitz and Richards (1955). Pigments were computed using the nomographs prepared by Duxbury and Yentsch (1956).

Every two hours during the day, additional aliquots were siphoned into four 150-ml. bottles, one of which was darkened with black tape. These were then suspended in the water, the three transparent bottles at depths of 0, 0.5 and 1.0 meter. After two hours, the bottles were removed and their dissolved oxygen concentration determined.

At high and low water each day, as determined from the tide gage, additional samples were collected from the surface and from a few centimeters above the bottom. These were returned to the laboratory where they were analyzed for salinity and used for total phytoplankton counts. At 10:30 on June 27 a single sample, taken from the whole water column, was frozen and subsequently analyzed for phosphorus and nitrogen fractions.[2]

Observations and Results

a. The phytoplankton

The phytoplankton population in Senix Creek consisted predominantly of centric diatoms, principally *Chaetoceros simplex*, *Thalassiosira nana*, and *Skeletonema costatum*. Other species present in abundance were the naviculoid diatom *Phaeodactylum tricornutum* (*Nitzschia closterium* forma *minutissima*), the green flagellate *Carteria excavata*, and the dinoflagellate, *Prorocentrum minimum*. The concentration of diatoms alone ranged from 61 to 109 million cells per liter. In addition there were observed large numbers of small coccoid cells, 1–2 μ in diameter, which were not identified but were either bacteria, blue-green or green algae. They bore some resemblance to the green alga, *Nannochloris atomus*, which was formerly present throughout Moriches Bay and its tributaries in concentrations exceeding 10^{10} cells per liter prior to the opening of Moriches Inlet in 1954. At that time the growth of *Nannochloris*, which virtually replaced the normal estuarine plankton flora, was attributed to high concentrations of pollutants originating from the duck farms, low salinities, and high temperatures (Ryther, 1954). These conditions still persist near the sites of pollution in the estuaries of Moriches Bay (for example in the Forge River and Seatuck Cove), where the phytoplankton was dominated by green algae and the water was a distinct green color in contrast to the rich brown color of the water in Senix Creek.

Table I

The vertical distribution of salinity and diatoms in Senix Creek

		Salinity (°/oo)	Diatoms (10^6/liter)
June 26	High tide		
	surface	11.94	92.8
	bottom	13.48	80.6
	Low tide		
	surface	10.22	109.0
	bottom	13.10	82.0
June 27	High tide		
	surface	11.73	82.8
	bottom	14.54	61.3
	Low tide		
	surface	11.64	79.5
	bottom	16.53	61.3

The small microorganisms in Senix Creek, though about ten times as numerous

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[2] Analyses were made by methods described in the following references: inorganic phosphorus (Robinson and Thompson, 1948); total phosphorus (Harvey, 1948); ammonia (Riley, 1953); nitrite (Rider and Mellon, 1946); nitrate (Mullin and Riley, 1955).

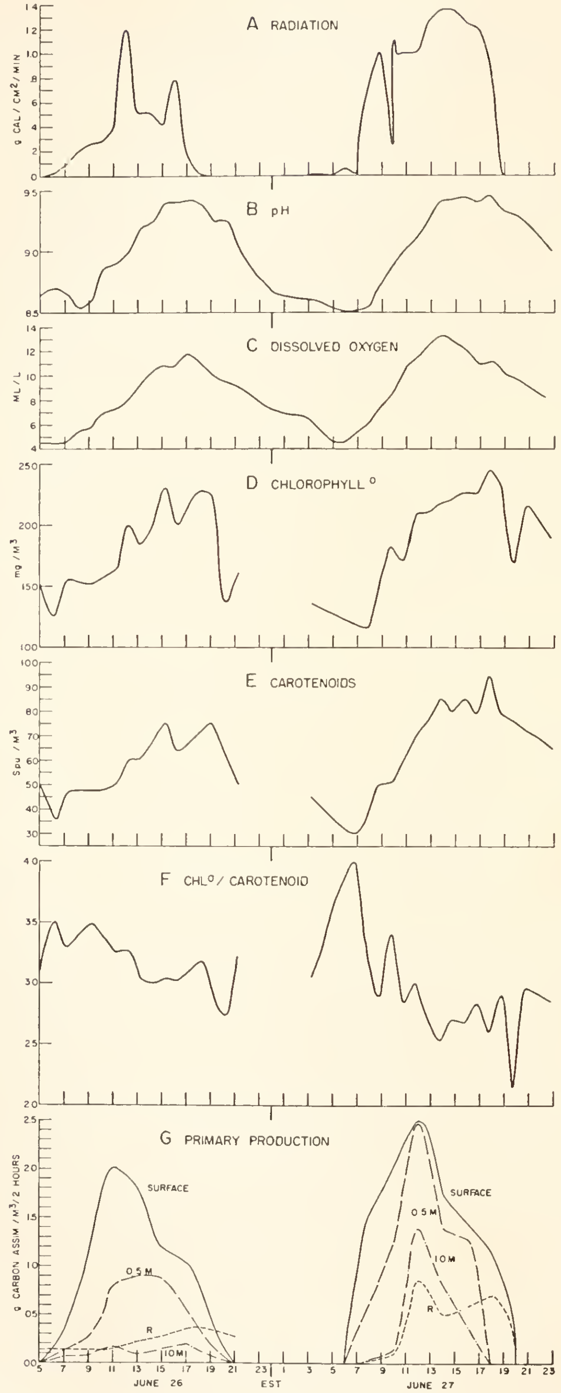

Figure 1. Variables measured in Senix Creek between 05:00, June 26 and 20:00, June 27.

as the diatoms, were probably insignificant in terms of total biomass since their cell volume is several hundreds of times smaller than that of the average diatom.

b. The physical environment

Figure 1A shows the incident radiation for the two days. The total daily radiation, obtained by integration of these curves, was 300 gram-calories/cm^2. on June 26 and 740 gram-calories/cm^2. on June 27.

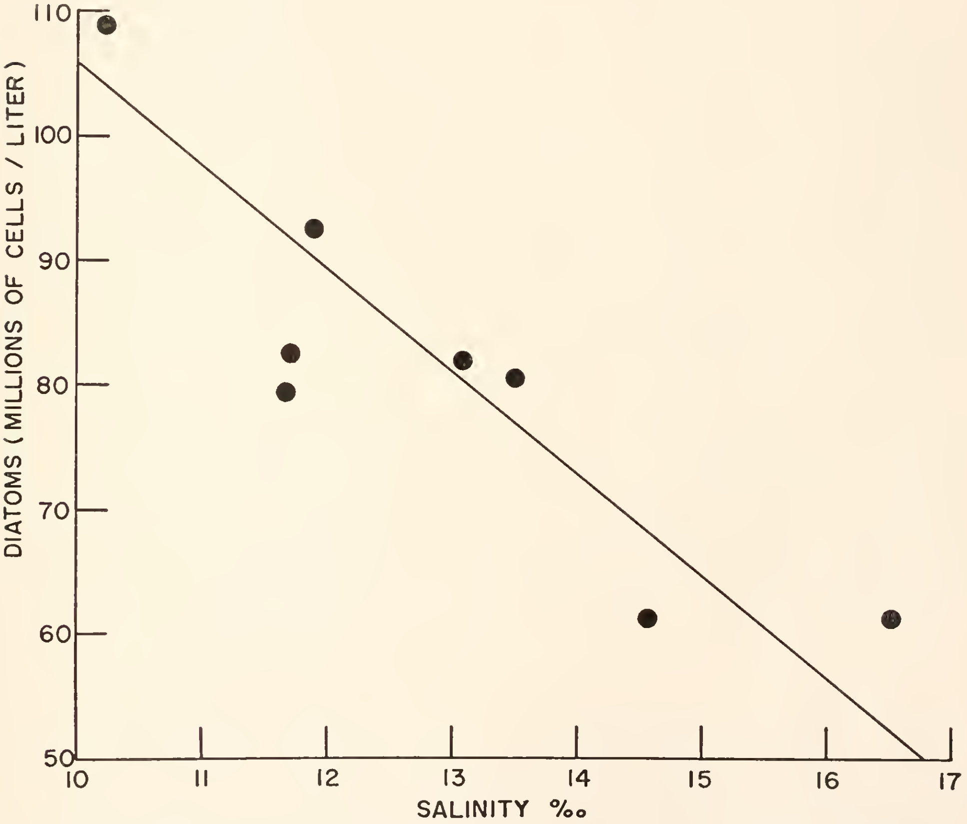

FIGURE 2. The relation between salinity and total diatom concentration, showing least squares line (R = −.875). Data from Table I.

The tidal influence in Senix Creek is extremely small. The range between high and low water on both days was approximately seven inches. Salinities ranged from 10–15 ‰ and showed no obvious correlation with the stage of the tide. However, salinities at the bottom were slightly higher than those at the surface, and salinities were higher the second day of observations than on the first (Table I).

Diatom counts were slightly lower at the bottom than at the surface and lower at both depths on the second day. Again there was no obvious relation to the tide, but there was a good inverse correlation ($r = -0.875$) between the diatom concentration and the salinity (Fig. 2). This correlation suggests that the diatom population did not change over the two-day period as a result of growth or death, but that the population was being diluted slowly with water from Moriches Bay, where the salinity ranged from 20 to 25 ‰ and diatom concentrations were generally less than one million cells per liter.

c. Dissolved oxygen and pH

Both pH and dissolved oxygen behaved in essentially the same manner, as may be seen by comparing Figures 1B and C. However, the high pH attained in the late afternoon of both days was maintained for several hours whereas the oxygen concentration reached its peak at the same time but then began to decline immediately. Water temperatures (which are not shown) ranged from 25° C. to 28° C. during the two-day period. Assuming a mean salinity of 12‰, the water was approximately 90% saturated with oxygen at daybreak, about 270% saturated at 14:00 on June 27. Despite this supersaturation, there did not appear to be a significant loss of oxygen to the air by diffusion since the decrease in oxygen concentration at night by respiration appears to have occurred at a constant rate. If appreciable loss by diffusion had occurred, this would have been dependent upon the oxygen concentration, and the decrease due to both causes (respiration and diffusion) would have been non-linear.

The pH reached a minimum of about 8.5 early in the morning and a maximum of almost 9.5 in the afternoon. Presumably at its maximum, no free CO_2 was available and any further photosynthesis was dependent upon bicarbonate or carbonate ions. Unfortunately no measurements were made of CO_2 in any of its fractions, nor may these values be calculated from pH, salinity, and temperature for these estuarine conditions as they may for either fresh or sea water. Again the regular behavior of the pH curve with time, shown in Figure 1C, indicates that CO_2 diffusion from air to water was negligible in comparison to the changes caused by photosynthesis and respiration.

d. Plant pigments

Figure 1D shows the concentration of chlorophyll *a* in the composite samples taken during the two-day period. Since the cell counts were not made on the same samples, it is not possible to represent chlorophyll on a cellular basis. The chlorophyll concentration in the water ranged from 116 to 245 mg./m^3., about a two-fold variability. Although the highest concentrations coincided on both days with low water, the connection between these factors is probably fortuitous. Certainly the variations in the pigment concentration are far greater than the observed differences in the diatom counts, caused by tidal fluctuations or otherwise. Despite the somewhat erratic distribution of the pigment concentration, it is still obvious that the chlorophyll increased gradually throughout the day, reaching its peak at about sunset, after which it decreased rapidly throughout the night until daybreak.

Similarly the plant carotenoid pigments increased during the day and decreased at night (Fig. 1E). Both chlorophyll and carotenoids increased to higher values on the second day, which differed from the first primarily in the amount of incident

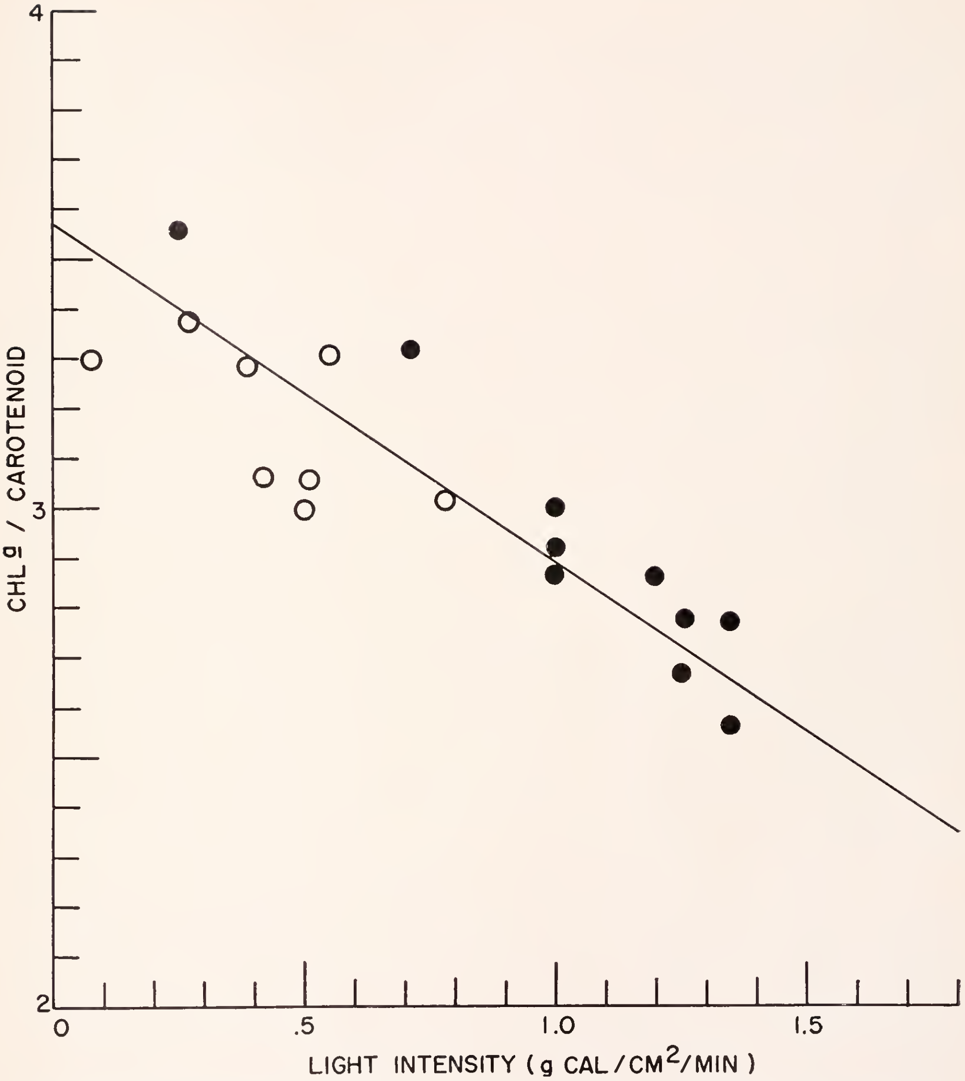

FIGURE 3. The relation between incident radiation and the ratio chlorophyll *a*:carotenoid pigments in the diatom population. Open circles, June 26. Closed circles, June 27.

radiation. The carotenoid pigments ranged on June 27 from a minimum of 30 SPU[3] at 05:45 to 95 SPU at 17:45, more than a three-fold variation.

The ratio chlorophyll *a*:carotenoid pigments (Fig. 1F) decreased throughout the daylight periods of both days from maximum values observed at sunrise, the more rapid decrease on June 27 again correlated with the greater incident radiation

[3] The spectrophotometric analysis of carotenoid pigments has not been standardized in absolute units and they are reported in specific pigment units after Richards with Thompson (1952). One SPU, however, is closely equivalent to one milligram of pigment.

on that day. Figure 3 shows the inverse relationship which was found between the intensity of solar radiation and the chlorophyll : carotenoid ratio. The variations in this ratio are the result of a differential effect of light on pigment synthesis and decomposition where the chlorophyll changes are of much greater magnitude than are the carotenoid changes (Yentsch and Scagel, unpublished). The significance and interpretation of the magnitude and changes in this ratio will be discussed in the final section of this paper.

c. Primary production

Primary production was calculated by the following three methods: (1) the *in situ* changes of oxygen in the water, (2) the "light-and-dark-bottle" oxygen measurements, (3) the "chlorophyll-radiation" method of Ryther and Yentsch (1957). The inability to measure or calculate total CO_2 prevented the use of pH changes or the C^{14} method for this purpose.

On June 26, the dissolved oxygen in the integrated sample collected over the one-meter water column increased from a minimum of 4.5 ml./liter at 07:00 hours to a maximum of 11.8 ml./liter at 17:00 hours, a difference of 7.3 ml./liter. If an assimilatory quotient of 1.25 is used, this change in oxygen is equivalent to a carbon fixation of 3.15 grams/m^2./day. As mentioned earlier, the decrease in oxygen at night appears to have been due almost entirely to respiration. This loss was equivalent to 0.5 ml. oxygen/liter/hour. During the 10 hours of daylight, if respiration occurred at the same rate, this would account for a total of 5.0 ml. O_2/liter or 2.15 grams carbon/m^2./day. Adding this respiratory loss to the observed net production of 3.15 grams carbon/m^2./day gives a total or gross production of 5.20 grams carbon/m^2./day.

In the same way production was calculated for June 27, the net change in oxygen being equivalent to assimilation of 3.8, the respiration loss 1.7 and the gross production 5.5 grams carbon/m^2./day.

The two-hour "light-and-dark-bottle" experiments which were described above were also used to calculate gross and net production. The differences between the oxygen concentration of the light bottles at 0, 0.5, and 1.0 meter and that of the accompanying dark bottle over the two-hour experimental periods, converted to carbon assimilation as above, are shown in Figure 1G. The carbon equivalent of respiration for the same two-hour periods was obtained from the difference between the oxygen content of the water at the beginning of the two-hour period and that of the dark bottle. These curves were integrated to obtain daily photosynthesis at each depth and daily respiration. These values in turn were plotted against depth and integrated to give daily photosynthesis and respiration beneath a square meter of surface. Gross photosynthesis for June 26 calculated by this method was 3.5, respiration was 1.7 and net photosynthesis 1.8 grams carbon/m^2./day. On June 27 the values were 6.2, 2.8 and 3.4 grams carbon/m^2./day for gross production, respiration and net production, respectively.

The *in situ* oxygen changes at night appeared to indicate a constant respiration rate of 0.5 ml. O_2/liter/hour, and this, as described above, was used to correct the net *in situ* change observed in daylight to give gross production. An examination of the two-hour bottle experiments during the day shows that, when measured in this way, respiration was by no means constant but varied roughly in proportion to the rate of photosynthesis. On June 27, for instance, the respiratory rate ranged from 0.06 ml./liter/hour in the early morning to about 1.00 ml./liter/hour at midday. These measurements, though somewhat crude, emphasize the need for a reconsideration of the tacit assumption made by most ecologists that respiration measured at night, or for long periods in dark bottles, is the same as that which occurs in the light in conjunction with photosynthesis.

The third method for estimating production is that developed by Ryther and Yentsch (1957). This method requires measurement of the concentration of chlorophyll *a*, the total daily incident radiation, and the extinction coefficient of visible light in the water. The latter was determined by the measurement of light penetration to one meter with a submarine photometer at 13:30 hours on June 26. The extinction coefficient (k) so determined was 4.0. Use of this method required an obvious over-simplification, since the chlorophyll *a* concentration, as has been pointed out, varied throughout the day. A mean value of 200 mg. chl*a*/m^3. was used for the calculation for both days, and this was assumed to be uniformly distributed over the one-meter water column. The resulting values for gross production were 3.2 and 5.1 grams carbon/m^2./day for June 26 and 27, respectively.

The results obtained by these three methods are summarized in Table II. They show rather good agreement except for the values obtained by *in situ* oxygen changes on June 25 which are almost twice as high as those obtained by the other two methods. It should be pointed out that the net production values which have been discussed refer to this process during the daylight hours only. The only estimates over a 24-hour period which can be made are based upon the *in situ* oxygen changes (and pH changes) which clearly reflect a net production for this period of zero. Finally, the net changes observed *in situ* and *in vitro* are acknowledged as representing changes brought about by the whole community including animals and bacteria, and do not characterize the plant population alone.

TABLE II

Primary production in Senix Creek on June 26 and June 27, as measured by three methods (grams carbon assimilated/m.²/day)

Method	Gross	Net (day)	Net (24 hrs.)	Gross	Net (day)	Net (24 hrs.)
In situ O_2	5.3	3.15	0	5.5	3.8	0
L-D bottle O_2	3.5	1.76	—	6.2	3.4	—
Chlorophyll	3.2	—	—	5.1	—	—

The efficiency of production on the two days may be roughly estimated by taking the median of the values obtained by the three methods for daily gross production, 3.5 and 5.5 grams carbon/m^2. on June 26 and 27, respectively. If the assumption is made that 50% of the photosynthetic production is carbon and has a heat of combustion of 5.5 k cal./gr. (see Krogh and Berg, 1931), and further that half the incident radiation may be used for photosynthesis, the efficiency may be calculated as:

$$\text{a) June 26} \quad \frac{3.5 \times 2 \times 5{,}500}{1{,}500{,}000} = 2.6\%$$

$$\text{b) June 27} \quad \frac{5.5 \times 2 \times 5{,}500}{3{,}700{,}000} = 1.6\%$$

f. The physiology of the bloom

There are several indications that the diatom population in Senix Creek was a non-growing one which had exhausted its supply of available nutrients and was able to subsist at a basal level, photosynthesizing just enough during the day to compensate for its metabolic requirement over a 24-hour period. This is best illustrated by the *in situ* oxygen and pH values, in which the net oxygen produced and CO_2 assimilated during the day are exactly compensated by the reverse processes at night. Further evidence of this is the fact that the concentration of diatoms remained unchanged over the 48 hours of observation except where such changes are attributable to tidal flushing.

The evidence that the bloom was nutrient-limited is somewhat sparse and indirect, but rather convincing. At 10:30 hours on June 27 an integrated water sample was collected and frozen. This was later analyzed for nitrogen and phosphorus fractions at the Woods Hole Oceanographic Institution. The results of these analyses are given below.

	μg Atoms/liter
$NO_2^- + NO_3^-$	3.40
NH_3^+	1.49
PO_4	3.80
Total P	16.0

A photosynthetic rate of 5.5 grams carbon/m².$/$day in a one-meter water column represents a requirement of 460 μgA carbon/liter/day. As Redfield (1934) and others have pointed out, marine phytoplankton assimilate carbon, nitrogen and phosphorus at an atomic ratio closely approaching 100 : 15 : 1. This rate of carbon assimilation is therefore equivalent to a daily requirement of 71 μgA/liter of nitrogen and 4.6 μgA/liter of phosphorus. Thus, the concentrations of these elements in the mid-morning of June 27 represented no more than a fraction of a day's supply of either nitrogen or phosphorus. These calculations were based upon the requirement of normal cells. Photosynthesis may of course continue after nitrogen and phosphorus are exhausted with the storage of carbohydrates and lipids. This is presumedly what was happening in this population, the cells using these stored materials to satisfy their metabolic requirements at night. Further studies of this type of population, with emphasis placed upon the diurnal cycle of nutrients, would be particularly interesting.

The behavior of the plant pigments is a further indication of the physiological condition of the population. The fact that both chlorophyll *a* and the carotenoids were synthesized during the day and decomposed at night signifies that the plants were drawing upon their cellular reserves to maintain themselves in the dark. When nutrients are available, this does not occur; in fact, chlorophyll may be synthesized in the dark under favorable growing conditions if the cells have sufficient respiratory reserves (Harvey, 1953).

Experiments in this laboratory (Ketchum *et al.*, 1958) and elsewhere have shown that both chlorophyll *a* and the carotenoid pigments decrease in diatoms in response to nitrogen, phosphorus or iron deficiency or excessive illumination. This nutritional chlorosis results in a more rapid decomposition of chlorophyll than carotenoid pigments. As the day progressed the pigment ratio decreased, presumably in part because of nutrient exhaustion which was hastened by greater demands of photosynthesis at high light intensities (Fig. 3).

The picture which emerges from these various bits of evidence, then, is that of a static diatom bloom of great magnitude, its nutrient supply exhausted or at least reduced to the level where growth could not occur. Yet it was not a dying population, except insofar as physical forces tended to disperse it. It was capable of carrying out organic synthesis at a rate some 10–100 times that of normal plankton communities, drawing upon these materials for its metabolic requirements much the same as a mature animal maintains a balance between its assimilation and metabolism.

It would appear, then, that populations of phytoplankton such as we have described here, though not actively growing, are not necessarily dying either. They are merely living in a different growth phase, a condition in which they may persist for long periods of time if they are not destroyed or dispersed by external factors. Perhaps in diatom populations, as elsewhere, the bloom of maturity may outlast the bloom of youth.

SUMMARY

1. A dense population of planktonic diatoms was studied over a 40-hour period in a small tidal creek on the south shore of Long Island, New York.

2. Measurements were made at frequent intervals of incident radiation, light penetration, salinity, temperature, dissolved oxygen, pH, concentration of diatom cells and their pigments, and dissolved inorganic nutrients. Photosynthesis and respiration were measured by oxygen changes in bottle experiments and estimated from *in situ* oxygen changes and from chlorophyll *a* and radiation.

3. The plankton community appeared to be nutrient-limited and consisted of a static, non-growing diatom population which was being slowly diluted by tidal action. This was indicated by the diatom counts, the behavior of their pigments (which increased throughout the day and decreased during the night) and the concentration of available plant nutrients.

4. Rates of primary production measured by three methods showed good agreement, the values ranging from 3.2 to 5.3 grams carbon assimilated/m^2./day on June 26, from 5.1–6.2 on June 27. Total incident radiation for the two days was 300 and 740 gram calories/cm^2./day, respectively, and the efficiency of the photosynthetic utilization of visible radiation for the two days was estimated at 2.6% and 1.6%, respectively.

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THE FORMATION OF SUBNUCLEAR AGGREGATES IN NORMAL AND SYNCHRONIZED PROTOZOAN CELLS[1]

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Since Bütschli (1876) established the nuclear dualism in ciliates, there has been much speculation about the biological role of the micro- and macronucleus. The mitotic behavior of the micronucleus, with its delicate apparatus for chromosomal segregation, led to the generally accepted view of the importance of the micronucleus in inheritance and reproduction. The macronucleus, on the other hand, which was found to divide "simply" by pinching in two, was considered to be concerned "only" with the regulation of metabolic functions in the cell.

This concept of the duality of nuclear function as formulated by Hertwig in 1889, was substantiated by Goldschmidt (1904) and Popoff (1908). These authors distinguished between the genetically-active idiochromatin and the trophochromatin, which was concerned exclusively with the cellular metabolism. In the uninuclear protists both types of chromatin were considered to be present in one nucleus, while in ciliates the idiochromatin was confined to the micronucleus and the trophochromatin was found in the macronucleus only.

The view of the dualistic function of the nuclei in ciliates was abandoned after experimental data accumulated showing the controlling role the macronucleus plays in the processes of cell division and regeneration (Grell, 1950). The genetic importance of the macronucleus in ciliates stimulated cytological studies of its structure. A considerable body of evidence has been accumulated during the past 30 years showing Feulgen-positive bodies in the cytoplasm, which could not be accounted for by "macronuclear fragmentation," the process of disintegration of the macronucleus upon conjugation of two cells. These bodies often have a spherical shape, and after Feulgen staining show the homogeneous appearance of micronuclei. Very often these bodies have been erroneously described as micronuclei, a fact which was pointed out by Kidder (1933). Diller (1936) observed simple fragmentations of the macronucleus in *Paramecium aurelia,* and he used the term "hemixis" to denote such autonomous changes of the macronucleus which are not related to sexual phenomena or binary fission.

It is believed now that the macronucleus of the ciliates consists of many diploid subnuclei (Sonneborn, 1947). We therefore propose the term "subnuclear aggregates" (SNA's) for the Feulgen-positive material lost or expelled from the macronucleus into the cytoplasm. The formation of SNA's may occur 1) by simple extrusion of Feulgen-positive material from the macronucleus, or 2) by loss during the process of binary fission of the macronucleus. An example of extrusion of chromatin masses from the macronuclear anlagen in the exconjugates of *Ancistruma isseli* was described by Kidder (1933), and a spontaneous "budding" of

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[1] This work was supported by grant G-2490 of the National Science Foundation.

macronuclei, independent of cell division, in *Ichthyophthirius multifiliis* was found by Haas (1933). In the course of binary fission of the macronucleus of *Colpidium colpoda* Kidder and Diller (1934) described how some of the nuclear material is left behind in the fission plane. This material becomes condensed and finally disappears. A similar phenomenon was described by Furgason (1940) in the amicronucleate strain T of *Tetrahymena pyriformis* and by McDonald (1958) in *Tetrahymena pyriformis* H.

These frequently observed chromatin extrusions from the macronucleus led Kidder and Diller (1934) to the suggestion of a presumptive role for this phenomenon. It was thought extrusion might be a manifestation of a universal principle of nuclear reorganization which, in turn, could account for a high division rate. However, no quantitative studies have so far been carried out on the formation of the SNA's, their frequency of formation, and their absolute size in various phases of population growth.

The system of synchronous cell division in *Tetrahymena pyriformis*, strain GL, as worked out by Scherbaum and Zeuthen (1953, 1955), was used for the study of this phenomenon. During the induced synchrony about 85 per cent of the cells are in the visible stage of fission and all stages of SNA formation can readily be found.

METHOD

The amicronucleate strain GL of *Tetrahymena pyriformis* was grown principally as described earlier (Scherbaum and Zeuthen, 1955). The growth medium was two per cent proteose peptone (Difco) with 0.5 per cent glucose and 0.1 per cent liver fraction L (Wilson Laboratories) in glass-distilled water. Salts were added as in the basal medium A of Kidder and Dewey, except that phosphates were omitted. The medium was filtered and autoclaved at 15 pounds for 15 minutes. One ml. of a three-day-old stock culture (approximately 2×10^5 cells per ml.) was used for the inoculation of 150 ml. of culture medium in a 500-ml. culture flask. The flask was submerged in a temperature-controlled water bath, which was mounted on a shaker.

Samples of 5 ml. were removed from the experimental flask at regular intervals for counting (Scherbaum, 1957) and for nuclear preparations. For the latter, the samples were concentrated by centrifugation in a hand centrifuge and the supernatant removed by suction. The concentrated cell suspension was fixed in one per cent aqueous osmic acid for two minutes. The cells were removed from the fixative by centrifugation, washed in water, and passed through alcohol (30 per cent to 100 per cent). The cells were then pipetted onto albuminized coverslips, slightly dried to affix the cells to the glass surface, and transferred to absolute alcohol for ten minutes. The coverslips were stored in 70 per cent alcohol. For the Feulgen reaction the samples were hydrolyzed in 1 N HCl at 60° C. for 12 minutes and exposed to the Schiff reagent for one hour.

RESULTS

At an approximate population density of 5×10^3 cells per ml. the first sample was removed. This served as the control for normal exponential multiplication. The second sample was removed during the synchronous division. For the induc-

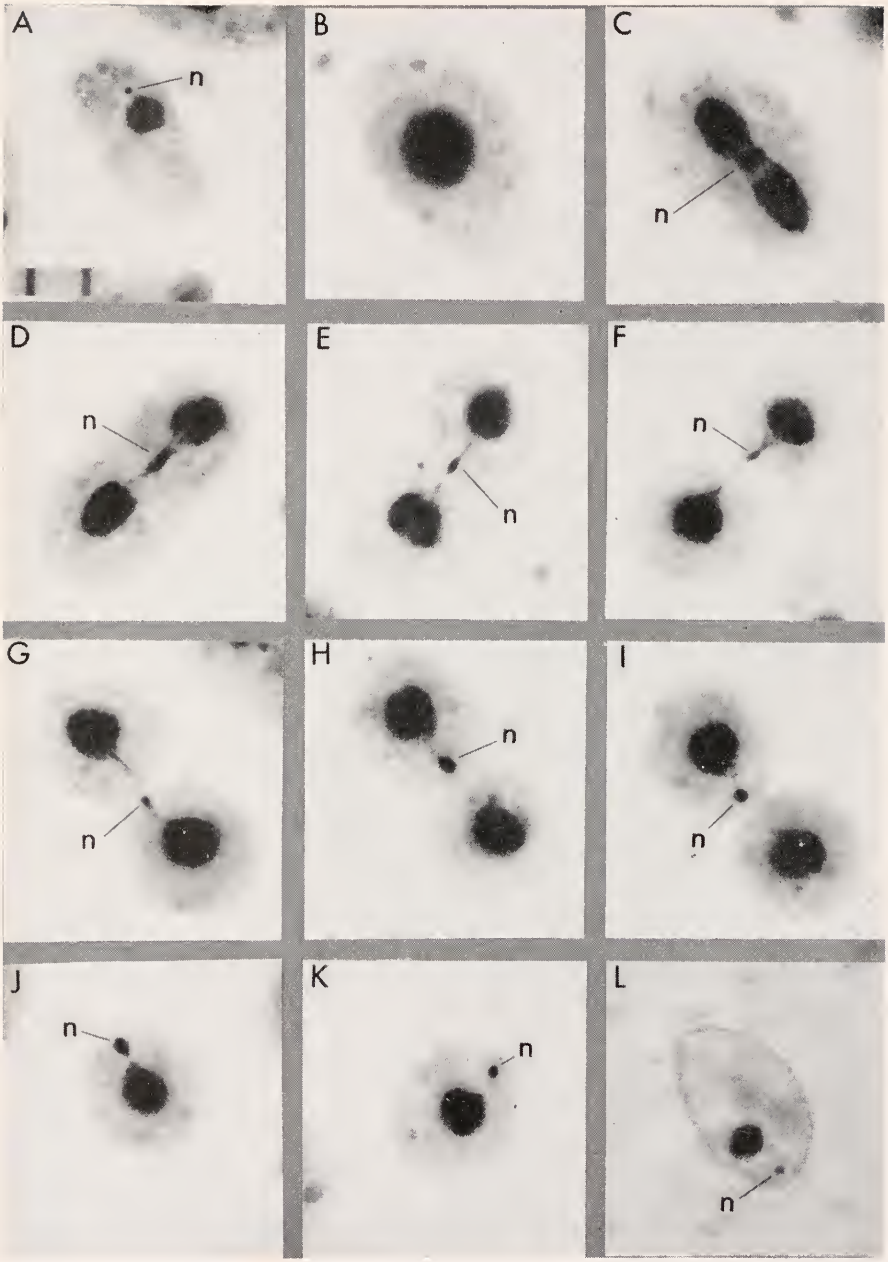

FIGURE 1. Photomicrographs of Feulgen-stained cells from various growth phases: Normal exponential multiplication (A), after temperature treatment (B), during and after the first synchronous division (C to K), and in maximum stationary phase (L). The distance between the two lines on the scale in (A) is 10 μ; "n" denotes a subnuclear aggregate (SNA). Further explanation in the text.

tion of synchrony the culture was exposed to seven temperature cycles. The temperature was changed every half hour between 28° and 33.9° C. One hour and 15 minutes after the end of the seventh cycle 80 to 85 per cent of the cells were in the visible stage of fission. The third sample was removed after 48 hours of subsequent growth when the cells were in the early stationary phase.

Figure 1 shows photomicrographs of cells in various stages of population growth and of the formation of the SNA's during division. In A the deeply stained SNA (n) is close to the macronucleus and resembles the micronucleus as shown by Holz, Scherbaum and Williams (1957) in mating type 1, variety 1, of *Tetrahymena pyriformis*. Figure 1, B shows a typical enlarged cell and nucleus after the end of the temperature treatment. No SNA from a previous division can be seen, although some were found in other preparations. In C the macronucleus elongates during the onset of synchronous division. A distinct portion of the nucleus seems to be "suspended" between the macronuclear halves pulling apart amitotically. In D to G this macronuclear remnant can be seen at various stages of cell division. In these phases of division the fragment still shows the typical granular composition of the macronucleus. However, somewhat later, when the fibrous connection between the macronucleus and the fragment disappears, the fragment tends to become spherical, the granular structure disappears, and the fragment becomes a dense homogeneous mass, resembling the micronucleus in this respect (I–L). Figure 1, J and K shows cells immediately after division. In cells of the early stationary phase of growth, SNA's were also found (L).

TABLE I

Size and number of subnuclear aggregates (SNA's) in various growth phases

Sample No.	Number of cells with SNA (%)	Mean macronuclear volume in μ^3	Mean volume of SNA in μ^3	SNA/macronucl. volume ratio in %
1 Control exponent. multiplication	16	265.0	1.9	0.72
2 Prior to synchr. division	22	1063.0	x	x
3 After synchr. division	55	430.0	12.2	2.84
4 Max. station. phase	6	122.0	1.8	1.48

In order to determine the average percentage cells with SNA's, 100 cells of each type were examined. The macronuclear volume for each growth stage is the average for the 100 cells measured. The mean volume given for the SNA is the average of 50 measurements; "x" denotes that no measurements were made.

For a quantitative estimation of the size and number of the SNA's the experimental culture was sampled in various growth phases. The result is shown in Table I.

The number of cells with SNA's is relatively constant in exponentially growing cultures (16 per cent) and increases slightly in the course of the heat treatment.

However, after synchronous division SNA's were found in 55 per cent of the cells. On the assumption that the SNA's observed in the cells prior to division are carried through the synchronous division step, one can calculate that in approximately 45 per cent of the cells undergoing division new formation of SNA's took place.

The mean volume of the SNA's is relatively constant in the logarithmic phase and stationary phase of growth. It is approximately 2.0 μ^3. This value is 0.7 per cent and 1.5 per cent of the macronuclear volume at these two growth phases, respectively. After the synchronous division the average SNA volume is 12 μ^3, showing a six-fold increase as compared to normal values.

Evaluation of the Results and Discussion

In almost all cells examined only one SNA was found, but in some cases two or three SNA's could be observed in one cell. From the frequency with which the SNA's occur at various growth phases it seems as if that they are broken down to Feulgen-negative material or are extruded from the cell. However, there is no evidence which might serve to evaluate either of these possibilities. The abnormally large nuclei of synchronized cells, together with the larger size of the SNA's of synchronized cells, might suggest that the size of the SNA's depends to some degree on the volume of the parent macronucleus. However, the size of the newly formed SNA's may vary, as can be seen in Figure 1, E–H. Furthermore, the size appears to be a function of the age of the SNA, since when first formed it is granular, similar to the macronucleus, and it then becomes homogeneous and smaller, apparently by condensation, before it disappears. In the present analysis we followed the formation of the SNA's with the Feulgen method for DNA only. However, nothing is known about the concentration of the basic proteins in these bodies. Basic proteins are normally found to be associated with

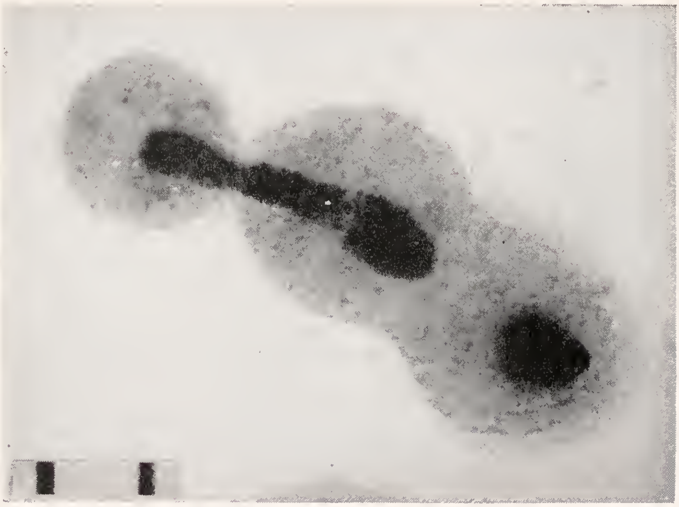

Figure 2. Photomicrograph of a Feulgen-stained cell during the first division after the heat treatment. The distance between the two lines on the scale is 10 μ. Further explanation in the text.

the DNA in the nucleus. A difference in stainability of the basic proteins in the micro- and macronucleus was observed by Alfert and Goldstein (1955) in mating types I and II of *Tetrahymena*. One could imagine that the original DNA basic protein ratio, as characteristic for the macronucleus and for the young SNA's in *Tetrahymena* GL, could change by preferential degradation of DNA in the course of the presumed condensation process occurring after formation of the SNA's.

Although the extrusion of macronuclear material in non-dividing cells and the formation of SNA's have been observed in various protozoan cells (see introduction), there is no conclusive evidence concerning the role which these phenomena play in the metabolism of the cells. Following the concept of strict equal distribution of parental DNA to the daughter cells one might be somewhat puzzled by this phenomenon. However, the high degree of polyploidy in the macronucleus suggests that such an equal distribution may not be a "*conditio sine qua non*."

A slight imbalance in timing of nuclear and cell division could cause this loss of DNA in the fission plane, and the failure for it to be incorporated into the daughter nuclei. In rare instances an "imbalance" of nuclear and cell division was observed in synchonized cells. For instance, Figure 2 shows a cell during synchronous division, dividing into three instead of two daughters. In the right part of the cell, nuclear division is completed, while cellular division lags slightly behind. In the left part of the cell the macronucleus is in division, while cytoplasmic division is far more advanced than in a normal cell with a comparable nuclear figure. That such irregularities hardly affect the viability of the cells is not surprising in view of the fact that the protozoan macronucleus is a highly polyploid system. Sonneborn (1947) concludes, from genetic evidence, that the macronucleus of *Paramecium aurelia* must contain about 40 diploid "subnuclei." These observations suggest the interesting problem of to what extent this high polyploidy of the macronucleus could be reduced experimentally. For instance, in starving cultures of *Tetrahymena pyriformis* strain S, Weis (1954) found a reduction in cell size to less than 10 per cent of the normal volume. These cells "regulated" back to their normal size upon addition of nutrients to the culture medium. If one assumes an almost constant nucleo/cytoplasmic ratio and 40 diploid "subnuclei" (as found for *Paramecium*), one might expect the starved cells to carry only 4 diploid "subnuclei."

Opposed to the view that the loss of subnuclei during binary fission is an arbitrary phenomenon, based on mere chance, is the idea which attributes a strict regulatory function to these processes. Findings by Kidder and Claff (1938) seem to substantiate this point of view. These authors investigated the life cycle of *Colpoda cucullus* and described chromatin extrusion following each division in regular and predictable fashion. This "budding" of the macronuclei occurs almost synchronously in the two daughter cells. In contrast to the loss of DNA *during* the fission process, as described for synchronized cells of *Tetrahymena*, we have in *Colpoda cucullus* an example of active regulation or reorganization of some sort *after* the daughter cells are formed.

The authors wish to thank Prof. W. H. Furgason, Department of Zoology, U.C.L.A., for his criticism and advice in preparation of the manuscript. Prof. W. Balamuth, Department of Zoology, University of California, Berkeley, Prof. Furgason and Dr. J. Loefer, O.N.R., Pasadena, kindly supplied samples of *Tetrahymena pyriformis*, strain GL.

SUMMARY

1. The formation of "subnuclear aggregates" (SNA's) is studied quantitatively in synchronously-dividing cells of *Tetrahymena pyriformis* strain GL.

2. In normal cultures approximately 16 per cent of the cells were found to contain SNA's. This value rises to 55 per cent after synchronous division. The SNA/macronuclear volume ratio is 0.72 per cent in normal cells and 2.8 per cent in cells after synchronous division.

3. The possible significance of the formation of SNA is discussed.

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STUDIES ON DIGENETIC TREMATODES OF THE GENERA GYMNOPHALLUS AND PARVATREMA

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Historical Review

The genus *Gymnophallus* was erected by Odhner (1900) to contain *Distomum deliciosum* Olsson, 1893 and other small species from the gall bladder, intestine and bursa Fabricii of shore-birds. Subsequent observations have shown that the asexual generations of these worms occur in bivalve mollusks and that the cercariae, which are produced in sporocysts, belong to the Dichotoma group of furcocercous larvae. Typically, these cercariae have eye-spots and short bifid tails, although either or both may be reduced or absent. *Cercaria dichotoma* emerges and swims as a furcocercous larva. In certain species the tail undergoes regression and is lost before the larva emerges from the sporocyst whereas, in others, apparently no tail is formed. On emergence from the first intermediate host, the cercariae attach to the mantle or body wall of bivalve or gastropod mollusks where as unencysted metacercariae, they develop to almost definitive size. The metacercariae may produce lesions on the mantles of their hosts and such injuries stimulate proliferation of tissues, especially of the secreting layer of the mantle, and deposition of nacreous material. Despite the observations of many investigators over a period of more than fifty years, no complete life-history has yet been worked out and the specific relations between particular cercariae, metacercariae, and sexually mature worms remain undetermined.

The presence of pearly formations in the mantle of *Mytilus edulis* has been known for at least three hundred years. According to Giard (1907), they were reported by Olaus Worm in 1655 from mussels taken at Roeskild, near Copenhagen. Robert Garner observed them in *M. edulis* from the English coast and he (1872) recognized that they were formed as a reaction by the mollusk to a small distome parasite on the mantle. Baron d'Hamonville (1894) found pearls (*sans valeur*) in *M. edulis* at Billiers (Morbihan) France, although the infection was limited to the area of the port. Giard (1897) reported small distomes, often associated with irregularly shaped calcareous deposits, between the mantle and shell of *Donax trunculus* L., *Tellina fabula* Gronov, *Tellina tenuis* DaCosta and *Tellina solidula* (= *T. balthica* L., *ex parte*) from Boulogne-sur-Mer. The worms were 0.5 mm. long, with rudiments of testes, but no ovary, and Giard suspected that they might be stages in the life-cycle of *Brachycoelium luteum* (van Beneden), a parasite of the common dogfish, *Scyllium canicula*. The larger specimens were often less active, more opaque, and filled with sporozoans (Glugidées). Dubois (1901) studied the parasites of *M. edulis* at Billiers and found them in reddish brown spots, which were the loci of pearl formation. The worms measured 0.4–0.6 mm. in length and for them he proposed the name, *Distomum margaritarum.* He found the same or a similar parasite in *Mytilus galloprovincialis* from the coast of Provence.

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Jameson also studied the parasites at Billiers; he (1902) described and figured the worms which he stated closely resembled *Distomum somateriae,* whose mature stages had been described by Levinsen (1881) from the intestine of the eider duck, *Somateria mollissima,* taken near Egedesminde, Greenland. He recalled that Möbius (1857) had reported a trematode associated with pearls in the pearl oyster, *Margaritifera margaritifera,* from the west coast of N. America. Following the action of Stossich (1899), Jameson referred the species from *M. edulis* to *Lecithodendrium* Looss, but misspelled the name, *Leucithodendrium.* He reported that he had found larvae, similar to those in *M. edulis,* in sporocysts in *Tapes decussatus* and that he had infected *M. edulis* with these sporocysts. The larvae in the sporocysts were tailless; they differed from those in *M. edulis* only in their smaller size, paler color, more distended excretory organs and empty gut, and in the possession of special sense organs and eyes. The sporocysts were present in all of almost 200 *Tapes* examined, located chiefly in the margin of the mantle where it is attached at the pallial line. The sporocysts were spherical to oval, about 0.5 mm. in diameter, with six to ten cercariae in each. Jameson also reported that *M. edulis* from Piel, near Barrow, Lancashire, England, were heavily infected with distome larvae, similar to those at Billiers, but that *Tapes* was not present at Piel. The sporocysts at Piel were found in *Cardium edule. Mytilus edulis* at Piel were exposed with specimens of *Tapes decussatus* from Billiers, with an apparent increase in the number of parasites. Returning to Billiers, Jameson found every specimen of *Oidemia nigra* heavily infected with *L. somateriae.* The worms were present throughout the length of the intestine. He described and figured the adult stage of *L. somateriae*; the worms were 0.2 to 0.55 mm. in length, about one-half the size of the larvae in *M. edulis,* and Jameson stated that the striking likeness except for size, between the larvae from *M. edulis* and *L. somateriae* and the occurrence of the latter in two species of birds that are known to feed *par excellence* on mussels is almost sufficient to prove their identity without the feeding experiment. He described the manner in which the mantle of *M. edulis* forms a sac around the trematode larva and deposits the nacreous material. Giard (1903) confirmed the observations of Jameson on pearl formation and, since pearls are the sarcophagi of trematode larvae, proposed their production by artificial means.

The first report of the asexual generations of *Gymnophallus* was an incidental footnote by von Siebold (1837) who found the sporocysts and cercariae in *Macoma balthica.* Lebour (1904) described the sporocysts in the liver and about the intestine of *Cardium edule* as (p. 83) "oval, pointed at one extremity, with two conspicuous black eyes, the other end is rounded and the whole body is covered with cilia which are constantly in motion in the living animal. The sporocyst may contain three different elements, (1) spherical masses full of smaller spheres, (2) the same but with two of these masses in one envelope, (3) minute sporocysts exactly like itself and containing small spheres." Miss Lebour regarded the "sporocysts" as the first larval stage of an echinostome species whose metacercariae she found encysted in the foot of the mollusk but whose cercarial stage was not discovered. In a footnote she added (p. 84), "since writing this I have discovered another stage of the worm in the liver of the cockle, of which particulars will be given later." In the report for 1905, Miss Lebour recognized that the "sporocysts" from the liver of *C. edule* were not related to the echinostome larvae in the foot, for which she had postulated the life-cycle. She stated that *C. edule* harbors the sporocyst stage of three species of trematodes; the one she reported in 1904, the second is "the sporocyst and cercaria stage of the 'pearl trematode' which is the cause of pearls in the mussel," and the third species was described as very contractile and of various shapes, without eyes and not ciliated. The report contains a brief description and figure of a "cercaria" from *Tellina tenuis* and *Donax vittatus* from the Alnmouth sands. From the figure, it is clear that the supposed "cercaria" is a gymnophallid metacercaria.

Johnstone (1905), in *C. edule* at Piel, found a fork-tailed larva which he described and figured as *Cercaria fissicauda* La Valette. It was probably not the tailless, ocellate species that had been reported by Jameson and was possibly *Cercaria dichotoma* Müller. This latter species, found free in the Mediterranean near Nice by Müller and described in La Valette (1855), was reported from the marine lamellibranch, *Scrobicula tenuis* by Villot (1875), but Villot (1878) assigned the larvae to *Cercaria fissicauda* La Valette, 1855. As noted by Pelseneer (1906), *C. fissicauda* was described from a fresh-water gastropod, *Lymnaea stagnalis*, and Villot's (1878) designation was obviously an error. The cercaria described and figured as *C. fissicauda* by Villot (1878) was identified as *C. dichotoma* by Pelseneer. Pelseneer described, as *C. dichotoma*, a species which he found in *Tellina solidula* from deep water near Boulogne-sur-Mer. The cercariae developed in short, nodose, colorless sporocysts and closely resembled the figures of *C. dichotoma* as given by La Valette and that of the species by Villot (1878) which he designated as *C. fissicauda*, but which he previously (1875) had identified as *C. dichotoma*. Pelseneer also referred to *C. dichotoma* an unnamed cercaria described by Huet (1888). Huet found this species in *Cardium edule* on the coast of Normandy and his description clearly contravenes its identity with *C. dichotoma*. According to Huet, the sporocysts were short, compact, spherical to pyriform, with the anterior end elongated in the form of a neck. The sporocysts were covered with cilia and swam freely. Immature sporocysts were 0.2 mm. in length and grayish in color; older ones, 0.3 mm. in length, were yellowish and the birth-pore, situated at the anterior end, was surrounded by five tubercules, below which there was a crown of verrucosities and the intervening area was covered with fine, stiff bristles. Mature sporocysts, 0.5 mm. long, were yellow and contained a few distomid cercariae with bifid tails, but on completion of development the tails were shed and the cercariae emerged from the sporocysts. The figures of Huet show that the cercaria is a gymnophallid larva, but it is not *C. dichotoma*, which emerges and swims as a furcocerous larva. Pelseneer also described a new species, *Cercaria syndosmyae*, from *Syndosmya alba* at Boulogne-sur-Mer. The sporocysts were 0.75 to 1.0 mm. in length, cylindrical, without constrictions. The cercariae were elongate, the acetabulum, near the middle of the body, was slightly larger than the oral sucker; the ceca were short, globose, and the cephalic glands were straight, moderately long. The furcae were longer than the stem of the tail. Young distomes, of corresponding morphology, were found often between the mantle and shell of *Donax* and other bivalves in the same locality. Odhner (1911a) postulated that *C. syndosmyae* is a larval stage of *Haplocladus minor*. This suggestion appears more credible since Cable (1953) included the Haplocladinae and Gymnophallinae as subfamilies in the family Fellodistomatidae.

Meanwhile Odhner had studied the trematodes of marine birds. He (1900) re-described and figured *Distomum deliciosum* Olsson, 1893, from the gall bladder of three species of *Larus*, which he named as type of a new genus, *Gymnophallus*. In the new genus he included *G. somateriae* (Levinsen, 1881) from the intestine of *Somateria mollissima*, *G. micropharyngeus* (Lühe, 1898) from the gall bladder of a North African flamingo, and two new species, *G. choledochus* from the gall bladder of *Vulpanser tadorna*, and *G. bursicola* from the bursa Fabricii of *Somateria mollissima*. In a later paper, Odhner (1905) re-described *G. somateriae* and commented on the findings of Jameson, in which the metacercariae from *M. edulis* were identified as larval stages of *G. somateriae*. Since the larvae from *M. edulis* were much larger than the sexually mature *G. somateriae*, Odhner contended that they could not be identical and that the material of Jameson represented two closely related species. The larger species, whose larvae induced pearl formation in *M. edulis*, was referred to *G. bursicola* since in morphology it agreed completely with the description of that species. The smaller mature worms from *Oidemia nigra*, according to Odhner, belonged to a different and unnamed species. The metacercariae described by Jameson from *M. edulis* agreed completely with others found by Odhner in the original material of *Saxicava rugosa* (= *Hiatella rugosa*) collected by Levinsen at Egedesminde, Greenland and which as noted by Levinsen, differed from *G. somateriae* in total size and size of suckers. Odhner noted differences between *G. somateriae* and the mature worms found by Jameson in *Oidemia nigra* at Billiers, which he postulated belong to a new and as yet unnamed species. Discussing the genus *Gymnophallus*, Odhner stated that it could not be included in any of the previously recognized subfamilies, and it was designated as type of a new subfamily, Gymnophallinae.

Nicoll (1906) examined *Cardium edule* at St. Andrews, Scotland; from May to July no infection was noted in several hundred individuals; later, sporocysts similar to those reported by Jameson in *Tapes* were found abundantly in the midline, dorsally, just over the posterior border of the liver. Individual sporocysts appeared as yellow spots of various sizes in an opaque, whitish mass which measured about four by three millimeters. Each sporocyst contained from two to fifty cercariae. The cercariae were tailless, 0.11 to 0.28 mm. in length, ocellate; the oral sucker (with minute papillae) about twice the size of the acetabulum which was situated just anterior to the bifurcation of the excretory vesicle. The testes were lateral, somewhat behind the acetabulum. Nicoll kept cockles (*C. edule*) and mussels (*M. edulis*) in the same tank for some time, but no infection of the mussels was noted.

Giard (1907) reviewed previous work on the trematodes which induce pearl formation in marine lamellibranchs; he reported that a worm, similar to the one described by Pelseneer, occurs frequently in *M. edulis* at Wimereux, near Boulogne-sur-Mer; that this species, named *D. margaritarum* by Dubois (1901) is probably identical with the one found in *Saxicava rugosa* by Levinsen and a stage in the life-cycle of *G. bursicola*. Giard also reported another and different distome which occurs abundantly "dans les *Donax* et les *Tellines* du Port de Boulogne" and which agrees with *G. somateriae* and differs from the parasite in *Mytilus*. *Oidemia nigra*, the pochard, was listed as the probable final host. Lebour (1905) had reported and figured a "cercaria" which occurred in *Tellina tenuis* and *Donax vittatus*, which she identified as the species described by Giard (1897) and regarded by him as possibly a larval stage of *Brachycoelium luteum*.

Nicoll (1907) described *Gymnophallus dapsilis* n. sp., from the bursa Fabricii of the scoters, *Oidemia fusca* and *Oidemia nigra*. This species differs from *G. bursicola* and other species of *Gymnophallus*, in that the vitellaria are anterior to the acetabulum. The worms were small, 0.84 to 1.13 mm. in length; however, Nicoll suggested that they may be adults of the species whose larvae occur in *M. edulis*.

Lebour (1908) described three gymnophallid cercariae (actually metacercariae): *C. glandosa* from *Paludestrina stagnalis* (a gastropod) which measured 0.2 mm. in length and had many large glands in the anterior part of the body; *C. macomae* from *Macoma balthica*, which measured 0.7 mm. long; and *C. strigata*, the species which Giard (1897) had regarded as the larva of *Brachycoelium luteum* and (1907) as that of *Gymnophallus somateriae*. Miss Lebour reported that it occurs very commonly in *Tellina tenuis* and rarely in *Donax vittatus* at Alnmouth. This is the same species reported by Lebour in 1905; the worms measured 0.3 to 0.4 mm. in length; the oral sucker was 0.09 mm. in diameter, and the acetabulum 0.05 mm. Miss Lebour noted that Pelseneer had described and figured what was evidently the same larva, and which he regarded as a later stage of the furcocercous cercaria named by him, *C. syndosmyae*. But in *C. syndosmyae* the suckers are nearly equal in size which renders their identity unlikely, and the larvae found in sporocysts in *Cardium edule* and *Tapes decussata* by Jameson, Nicoll and Lebour were tailless. Miss Lebour stated that *C. strigata* is broader than *C. somateriae* and the striations produced by the rows of spines are more conspicuous. Furthermore, ducks seldom feed at Alnmouth, and the adult stage of these parasites is probably in gulls; possibly it is *G. deliciosus*. Miss Lebour also described a larva which she identified as *Cercaria dichotoma* Müller, from the small bivalve, *Scrobicularia tenuis*. The figure shows that it is the cercarial stage of a species of *Gymnophallus* and Miss Lebour believed that the same species had been recorded by Pelseneer from *Tellina solidula* (= *Macoma balthica*), by Johnstone from *Cardium edule* on the Lancashire coast, and by Huet in *C. edule* in Normandy.

Lebour (1912), in a review of the British marine cercariae, recognized five gymnophallid cercariae (actually metacercariae): (1) *Cercaria margaritae*, the pearl trematode of Jameson (1902), which occurs in sporocysts in *C. edule* and *Tapes decussata*; (2) *Cercaria scrivenensis* sp. inq., based on two specimens from *Tapes pullastra* at Loch Scriven, the Clyde, but not well characterized; (3) *Cercaria glandosa* Lebour, 1908; (4) *Cercaria macomae* Lebour, 1908; and (5) *Cercaria strigata*, Lebour, 1908. Apparently Miss Lebour was not yet aware that the larvae she regarded as cercariae were really unencysted metacercariae, since she noted that *Cercaria dichotoma*, which she described and figured, is closely allied in structure to *Gymnophallus* but, because of its forked tail, she placed it in a separate group. *Cercaria margaritae* has eye-spots and according to Jameson develops in sporocysts in the edges of the mantle of *Tapes decussatus* and *Cardium edule* whereas Nicoll and Lebour reported this species from sporocysts in jelly-like masses under the umbo of *C. edule*. These differences suggest that two distinct species were regarded as identical.

Sinitsin (1911) described sporocysts from *Syndosmya alba* of the Black Sea as *Cercaria discursata* n. sp. The sporocysts were simple, saccate, in the liver and gonad; the cercariae 0.06 mm. long with a bifid tail somewhat longer, normally shed in the sporocyst, but used in sluggish swimming when the cercariae are released from the sporocyst. According to Sinitsin, some of the larvae remain as unencysted metacercariae in *S. alba*, while others leave and migrate to various species of mollusks. They attack the tissues but do not encyst and increase about four times in size. In the same paper Sinitsin described *Adolescaria perla* from various mollusks, mostly near the gill plates of *M. edulis* and unidentified species of *Venus*. From the descriptions and figures of Sinitsin, both of these species can be identified as gymnophallids, and indeed, they may be stages in the life-history of a single species.

Stafford (1912) reported sporocysts and cercariae in *Mya arenaria* of the Gaspé Bay region of Canada. The venter of the clam was distended, soft, translucent, pale greenish yellow in color with hundreds of sporocysts, each containing about twenty fully-formed, forked-tailed cercariae; the body of one cercaria measured 0.138 by 0.082 mm. From the surface of the mantle of *M. arenaria* and *M. edulis*, Stafford reported metacercariae which measured 0.078 by 0.056 mm. These specimens were similar to those reported by Levinsen from the intestine of *Somateria mollissima* which occurs on the Gaspé, but since they were smaller than the cercariae from *M. arenaria*, Stafford concluded that they did not belong to the same species.

Jameson and Nicoll (1913) reviewed the question of pearl formation in *M. edulis* and the identity and life-history of the trematode larvae which are the inciting agents. Jameson's earlier experiments were not controlled; the form described by him (1902) as *G.* (*Leucithodendrium*) *somateriae* was now described as *Gymnophallus oedemiae*. Since the gravid worms are smaller than the metacercariae in *M. edulis*, these authors reasoned that they cannot be the adult stage of that species. They stated that the metacercariae in *M. edulis* may be *G. bursicola* as believed by Odhner or possibly *G. dapsilis* Nicoll. They postulated that the sporocysts and cercariae in *Tapes decussatus* are *G. bursicola*; that those in *C. edule* are *G. dapsilis*; and that metacercariae of both may occur in *M. edulis*. Although specimens of *Oidemia nigra* were naturally infected with a species of *Gymnophallus*, metacercariae from *M. edulis* did not develop in *Oidemia nigra* or *Fuligula* (*Nyroca*) *ferina*. Jameson and Nicoll recognized six species; *G. bursicola* Odhner; *G. dapsilis* Nicoll; and four new species; *G. oedemia*, *G. affinis*, *G. macroporus* and *G. ovoplenus*, all from the intestine of *O. nigra*.

Dollfus (1923) studied the metacercariae from *Mytilus galloprovincialis* described earlier by Dubois (1901). He found the worms to measure 0.23 to 0.27 mm. in length, with the oral sucker about twice the size of the acetabulum. They were similar to the *Adolescaria perla* of Sinitsin and to others which Dollfus had observed between the mantle and shell of *Tapes pullastra* on the English channel. For them he proposed the name, *Gymnophallus duboisi* n. sp.

Odhner (1900) had described *Gymnophallus choledochus* n. sp. on the basis of sketches made by Jägerskiöld, of a single specimen (then lost) from the gall bladder of *Vulpanser tadorna*. He (1905) re-described the species from specimens taken from the gall bladder of *Somateria mollissima* and *Somateria spectabilis* from East Greenland. The worms were pyriform, 0.9 to 1.1 mm. long, 0.35 to

0.50 mm. wide, with the acetabulum near the middle of the body, ceca which extended to the middle of the body, and testes lateral, postcecal. Isaitchikow (1924), apparently unaware of Odhner's re-description, reported *G. choledochus* from the gall bladder of *Aytha* (*Nyroca*) *ferina* from the Crimea. He noted that the distribution of the species is not restricted to Scandinavia, but extends to the Black Sea. He found that the coils of the uterus may fill the body from the oral sucker to the reproductive glands, that the topography of the genital organs is not fixed, the variations are numerous and very inconstant; eight different types were depicted by figures.

Palombi (1924) re-described *Cercaria margaritarum* Dubois, 1901 (actually a metacercaria) and stated that *G. duboisi* Dollfus, 1923 is a synonym. Later Palombi (1934) described and figured the metacercariae of *Gymnophallus strigata* (Lebour) and *Gymnophallus megacoela* n. sp. In an article by Fujita (1925), Dollfus described *Gymnophalloides tokiensis* n.g., n. sp., a gymnophallid metacercaria from the surface of the mantle of the Japanese oyster, *Ostrea gigas*. The text was translated by R. Ph. Dollfus, who added notes, diagnoses, and a bibliography. In this species the genital pore is located some distance anterior to the acetabulum and for it Dollfus provisionally proposed a new genus, *Gymnophalloides*. In the paper, Dollfus described a second species, *Gymnophalloides tapetis*, a metacercaria from *Tapes pullastra* at Saint Vaast-la-Hougue (Manche), which he noted is very similar to *Adolescaria perla* Sinitsin.

Cole (1935) reported an "orange sickness of mussels" at the Conway station on the coast of North Wales. According to him (p. 276), "The infected specimens were found without difficulty as the mantle is a vivid marigold to blood-orange due to the presence of innumerable sporocysts." The sporocysts were present also throughout the body of the mollusk. They were oval, bright orange, thin-walled, moderately contractile and measured about 1.1 mm. by 0.45 mm. They contained tailless cercariae which were described as a new species, *Cercaria tenuans*. The cercaria measured up to 0.3 mm. in length when extended; the oral sucker was 0.05 mm. and the acetabulum 0.07 mm. in diameter. The cercariae were without spines and died soon after emergence, which indicates an immature condition. In one mussel, Cole reported similar sporocysts in the digestive gland, but they contained daughter sporocysts and no cercariae. This observation led to the suggestion that *M. edulis* acquires the infection by way of the intestine. Cole predicated that the species described by him is probably identical with the one reported by Miss Atkins (1931) in 2.16 per cent of over ten thousand mussels from the estuary of the Camel, near Padstow.

Young (1936) described fork-tailed cercariae taken in a tow net in the Bering Sea, 15–65 kilometers from the nearest land. The larvae were without eyes or spines, 0.4 mm. long; the oral sucker was 0.085 mm. and the acetabulum 0.09 mm. in diameter. The specimens were reported to agree best with *C. syndosmyae* Pelseneer, and they may be a species of *Gymnophallus*.

Markowski (1936) described *Cercaria baltica*, a furcocercous species from sporocysts in *Macoma balthica*. The sporocysts were long, cylindrical, whitish, slowly motile; 1.2 by 0.15 mm., each contained from two to fifty cercariae. The cercariae measured in microns; body length, 133; tail-stem, 90; furcae, 38; oral sucker, 11; acetabulum, 44 by 30; cecal length, 52. The measurements as given do not agree with his Figure 6, in which the oral sucker is approximately the same size as the acetabulum. In *C. baltica* the entire surface of the body, including the tail-stem and furcae, is spined, there are four pairs of glands near the oral sucker, and the ceca extend to the anterior edge of the acetabulum which is slightly behind the middle of the body. Markowski recognized four species in the Dichotoma group of cercariae; *C. dichotoma* Müller, *C. syndosmyae* Pelseneer, *C. discursata* Sinitzin, and *C. baltica* n. sp. From the same host Markowski described two metacercariae: *Metacercaria morula*, in which a large number of larvae were enclosed in a single cyst; and *Metacercaria mutabilis* which are about three times as large as the former species and occur unencysted on the mantle or in folds of it.

Cole (1938) reported a brown mass below the hinge of *C. edule*, situated in a wedge-shaped cavity which, when torn, liberated thick-walled, colorless, immobile sporocysts and brown granules. The tailless cercariae, named *C. cambrensis*, were spined and measurements in millimeters were: length, 0.27–0.32; oral sucker, 0.04; acetabulum, 0.03; pharynx, 0.015. The acetabulum was about one-third of the body length from the posterior end; the ceca were reported to extend to the level of the posterior border of the acetabulum but are preacetabular in the figure. The cercaria was similar to *C. margaritae* of Jameson (1902) as described by Lebour (1912) and Jameson and Nicoll (1913), but differed in the absence of eye-spots and sensory papillae.

Rees (1939) re-described *Cercaria strigata* Lebour, 1908, which was actually a metacercaria found by Miss Lebour in *Tellina tenuis* and *Donax vittatus* at Alnmouth, Northumberland. Presumably it was identical with the unnamed larva found by Giard (1907) from the same hosts. Miss Rees found the cercariae in thin-walled, colorless sporocysts in the digestive gland of *C. edule*. The cercariae were not described other than the statement that they were the same as the metacercariae. The excretory vesicle extended to the region of the oral sucker and the anterior ends were bifid. The flame-cell formula was $2[(2+2)+(2+2)]$. *Cercaria strigata* was distinguished from *C. margaritae* of Jameson by absence of eye-spots and bristles and location of sporocysts, which in the former were just below the hinge. It differs from *C. cambrensis* which develops among brown granules in thick-walled sporocysts. According to Rees, *C. strigata* could be the larva of *Gymnophallus macroporous* Jameson and Nicoll, 1913 from *Oidemia nigra* but more likely is the larva of *G. deliciosus* which was found abundantly in the gall bladders of gulls in the region. She suggested that *C. cambrensis* is the cercarial stage of the metacercaria in *M. edulis* and identical with *Gymnophallus margaritarum* (Dubois).

Yamaguti (1939) described *Gymnophallus macrostoma* from the intestine of *Melanitta (Oidemia) nigra americana* (Swainson) from Korea. According to Yamaguti, the new species differs from *G. affinis* and *G. macroporus* in size of eggs and position of the ovary; it resembles *Gymnophalloides tokiensis* Fujita, 1925, a metacercaria, but identity must await experimental evidence. Yamaguti re-described *G. bursicola* from *Melanitta fusca stejnegeri* and *Melanitta nigra americana*; variations in morphology led him to regard *G. dapsilus* Nicoll as probably identical with *G. bursicola*.

Ogata (1944) found metacercariae in *Paphia (Ruditapes) philippinarum*, *Laternula kamakurama* and *Tellina* spp. which were raised to adults in cats and mice. The mature worms were identified as *G. bursicola*.

Uzmann (1952) described *Cercaria myae* from the gonads and digestive gland of *Mya arenaria* at Newburyport, Massachusetts. The sporocysts were clavate, unpigmented, motile, with thin walls and apical birthpores; they measured 0.21 to 0.60 mm. in length. He suggested that this is the same species reported by Stafford (1912) from the same host in Canada. The body of the cercaria is 0.12 to 0.25 mm. long; the tail-stem is about one-third the body length with furci slightly longer than the stem and spined at the tips.

Dubois, Baer and Euzet (1952) described *Cercaria mathiasi*, a furcocercous cercaria from the Mediterranean, which was identified as a species of *Tergestia*. The cercaria was included in a group with *Cercaria haswelli* Dollfus, 1927, which Odhner (1911b) had recognized as a species of *Tergestia*, and *C. dichotoma* La Valette. Although *C. dichotoma* has been widely accepted as a gymnophalline larva, the relation to *Tergestia* does not appear unlikely since Cable (1953) has included the Haplocladinae (which includes *Tergestia*) and the Gymnophallinae in the family Fellodistomatidae.

Hutton (1952) studied the gymnophallid parasites of *C. edule* at Plymouth, England. He described a new species, *Cercaria fulbrighti*, which developed in motile, irregularly shaped sporocysts with birthpores at the tips of snout-like protrusions. The sporocysts were found in the digestive gland, gonad, and dorsal part of the foot. Young cercariae in sporocysts have forked tails, which degenerate when the larvae are about one-half grown. The cercariae lack eye-spots, are spined, while the excretory vesicle extends parallel to the ceca and is not lyre-shaped. The mature cercaria agrees closely with *G. choledochus* and differs from *C. margaritae* which has eye-spots and in which the excretory vesicle is lyre-shaped and extends to the oral sucker. He reported that the excretory system has thirteen flame-cells on each side of the body. It is possible that the cilia in the common collecting duct were counted as the thirteenth flame-cell. Hutton differentiated between *C. fulbrighti* and *C. cambrensis* which he found in the same host species. He noted resemblances between *C. fulbrighti* and *C. dichotoma* as described by Pelseneer (1906) and Lebour (1908), but certain differences led him to regard them as distinct species.

Hutton (1953) described *Cercaria reesi* from *Hiatella arctica* and *Hiatella striata* taken at Drake's Island and Plymouth Sound. The sporocysts were ovoid to sausage-shaped, 0.32 to 0.80 mm. long and 0.196 to 0.352 mm. wide. The cercariae were fork-tailed, non-oculate, with two pairs of penetration glands. The flame-cell formula was $2[(2+2)+(2+2)]$, a total of 16 cells, the same as Rees (1939) had found in *C. strigata*. The oral sucker was surrounded by two pairs of protrusible spines and six tubercules. Hutton stated that *C. reesi* closely resembles *C. discursata* Sinitsin and *C. myae* Uzmann, 1952, and that all are probably members of the genus *Gymnophallus*.

Brinkmann (1956) in a study of the Trematoda of Iceland, reported a single unidentified specimen of *Gymnophallus* from the gall bladder of *Somateria mollissima* and another from the gall bladder of *Clangula hyemalis* which he described as a new species, *Gymnophallus bilis*. He noted that except for the location of the genital pore the latter worm agrees entirely with Levinsen's (1881) description of *G. somateriae*, which differs in essential respects from Odhner's re-description of that species, based on new material and possibly on a different species of *Gymnophallus*.

Gymnophallid trematodes have been known for more than a century. The first of these worms to be discovered were metacercariae from the mantle of *Mytilus edulis*, and sporocyst stages were reported by von Siebold (1837) in *Macoma balthica* from the coast near Danzig. Sexually mature specimens were described and named by Levinsen (1881) from the digestive tract of eider ducks, *Somateria mollissima*. Subsequently, worms have been reported from the gall bladder, intestine, ceca, and bursa Fabricii of different species of birds. Twelve specific names have been applied to these sexually mature specimens but the species are not clearly distinguished and the validity of certain of them is doubtful. During this time, cercarial stages have been described from a variety of bivalve mollusks, but frequently cercarial and metacercarial stages have been mistaken for one another and there is no reliable information to identify any cercaria with its metacercarial or adult stage. Metacercariae occur frequently in bivalve and rarely in gastropod mollusks, but knowledge of their previous larval or final sexually mature stages is completely lacking. The situation is chaotic and one of utter confusion.

Even the systematic position of the subfamily Gymnophallinae is equivocal. It was erected by Odhner (1905) who postulated relationship to the Heterophyidae but admitted (p. 314), "Die Frage, wo diese Unterfamilie zu placieren ist, kann dagegen nur der Gegenstand sehr unsicher Vermutungen sein." Fuhrmann (1928) included it in the family Acanthostomidae; Dawes (1946) in the family Microphallidae; and Uzmann (1952) suggested its probable relationship to the family Brachylaemidae. Cable (1953) transferred the Gymnophallinae to the family Fellodistomatidae, in the superfamily Brachylaemoidea. This action was based on the discovery of the first life-cycle in the subfamily, that of *Parvatrema borinqueñae*, a new genus and species from Puerto Rico. The unencysted metacercariae from the snail, *Cerithidea costata*, developed to maturity in baby chicks and since sandpipers, plovers, terns and herons of the region did not harbor gymnophalline trematodes, Cable postulated that the natural hosts were migrant ducks. Small, furcocerous cercariae, produced in sporocysts in *Gemma purpurea*, were recognized as the antecedent larval stage of the species. In the same paper, Cable suggested that *Metacercaria morula* of Markowski (1936) may be a cercaria, and that *Cercaria baltica* may be conspecific with *Metacercaria mutabilis* from the same host. This species is probably the one found by von Siebold in the same host a century before.

Present Project

For some years the U. S. Fish and Wildlife Service has been concerned with the attrition in stocks of the soft-shelled clam, *Mya arenaria*, along the New England coast. Reduction in numbers of these clams was reported by Smith (1950), Smith and Chin (1951), and Glude (1955). The junior author began a study of the significance of parasitism in relation to this problem at Newburyport, Massachusetts and continued it at Milford, Connecticut. He (Uzmann, 1952) published a description of the sporocysts and cercariae found in *M. arenaria* at Newburyport, Massachusetts. The larvae, named *Cercaria myae*, emerge from the sporocysts and swim in the sea; they have forked tails but no eye-spots. The amount of living material was limited and no infection experiments were attempted. Uzmann also found sporocysts and cercariae in *Hiatella arctica* of the Boothbay Harbor area of Maine, but they were not reported. In addition, Uzmann (1953) described

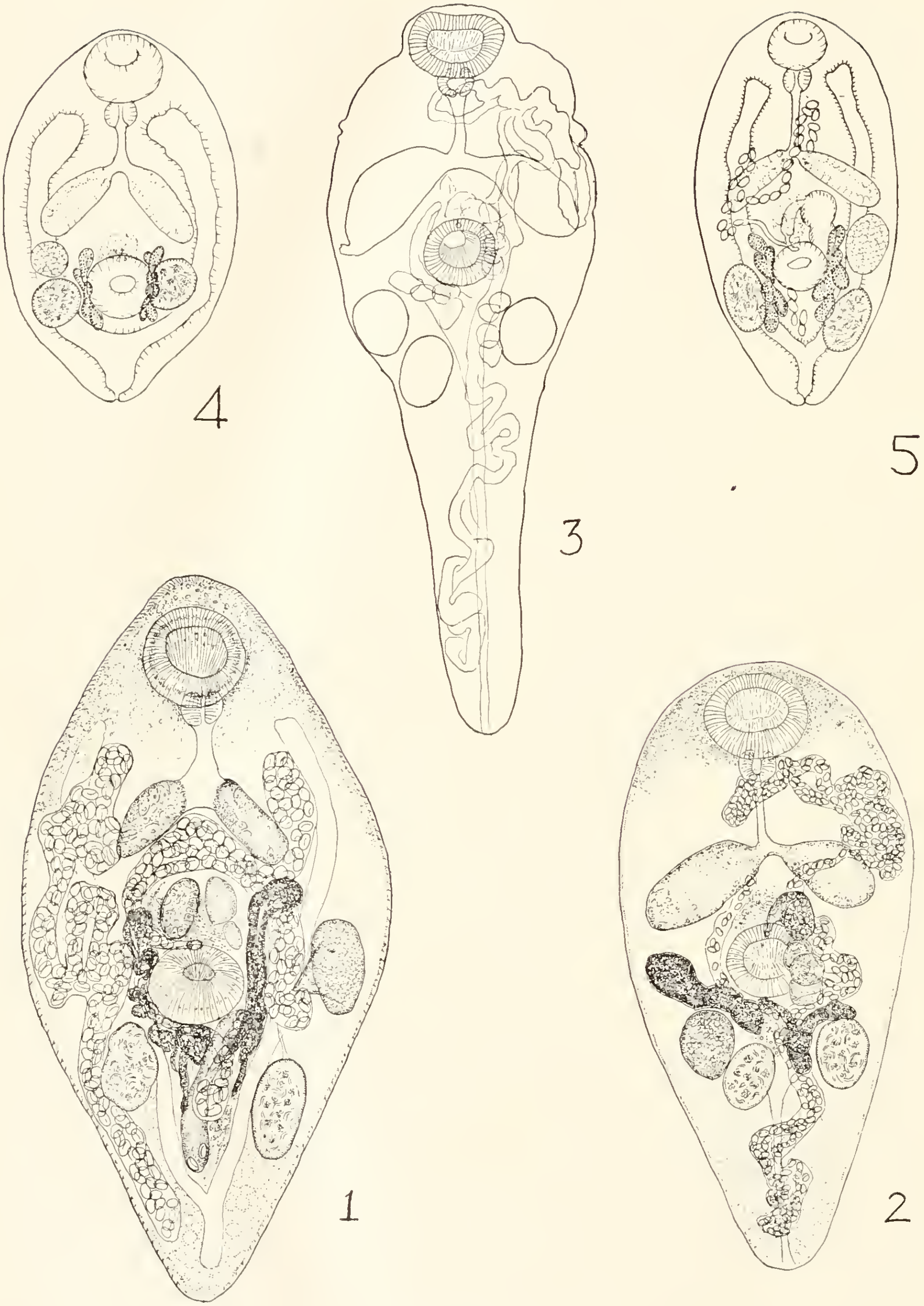

PLATE I

sporocysts and cercariae from *Mytilus edulis* taken in Long Island Sound, New York; this species, named *Cercaria milfordensis*, was referred tentatively to the genus *Proctoeces*. Metacercariae had been found in lesions, and associated with pearly deposits, in the mantle of *M. edulis* collected in the region of Newburyport, Massachusetts.

In collaboration with the senior author, attempts were made to determine the adult stage of the metacercariae from *M. edulis*. Specimens were fed on February 24, 1951 to a hamster and the animal was sacrificed on March 3, 1951. Sexually immature worms (Fig. 4) were recovered, which manifested diagnostic characteristics of the genus *Gymnophallus* and it appeared that the natural definitive hosts were mollusk-eating birds. Accordingly, metacercariae (Fig. 9) were fed to domestic chicks and ducklings, but no infection was obtained. Eggs of eider ducks, sent from Boothbay Harbor, Maine, were incubated in the laboratory at New York University and metacercariae, fed June 1, 1951 to recently hatched eider chicks, developed during ten days in the intestine to sexual maturity (Fig. 5). These worms belong to the genus *Gymnophallus*, but because specific descriptions are so inadequate, identification is tentative. Subsequently, the junior author was transferred to the Seattle, Washington, Laboratory of the U. S. Fish and Wildlife Service and the senior author, on retirement from teaching at New York University, was assigned to a study of the parasites of clams and of their predators. The green crab, *Carcinides maenas*, an important predator, harbors the metacercarial stages of a digenetic trematode whose life-history was worked out (Stunkard, 1957) and the adults were identified as *Microphallus similis* (Jägerskiöld, 1900). Returning to the investigation of the sporocysts and cercariae in *M. arenaria*, a study of the gymnophallid trematodes was resumed. For whole-hearted cooperation and for material, we are indebted to Walter R. Welch, Chief of Clam Investigations, Boothbay Harbor, Maine and members of his staff. In the summer of 1957 sporocysts and cercariae were found by the senior author in the digestive gland of *Gemma gemma* taken at Boothbay Harbor and unencysted metacercariae from the mantle of *G. gemma* developed to sexual maturity in the intestine of *Somateria mollissima*.

Most of the previous work on gymnophallid trematodes has been done in Europe; the parasites have been reported from different locations in different hosts, but specific determination on the basis of existing descriptions is virtually impossible. A satisfactory solution of the difficulties requires controlled experiments to discover and relate successive stages in the life-cycles of individual species. Certain questions are pertinent to a consideration of the problem of specificity: (1) to what extent can one species of *Gymnophallus* infect different primary, secondary, and definitive hosts; (2) are the gymnophallids in gulls, eider ducks, and

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EXPLANATION OF PLATE I.

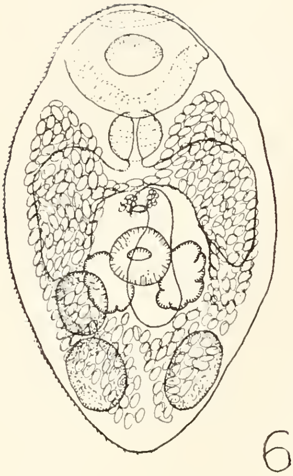
FIGURE 1. Adult from the bursa Fabricii of *S. mollissima*, Boothbay Harbor, Maine. Natural infection, specimen 1.0 mm. long, fixed, stained and mounted, ventral view.

FIGURE 2. Adult from the gall bladder of *S. mollissima*, Boothbay Harbor, Maine. Natural infection, specimen sketched alive, 1.3 mm. long, dorsal view.

FIGURE 3. Same specimen shown in Figure 2, fixed, stained and mounted, 1.7 mm. long.

FIGURE 4. Immature specimen from the mantle of *M. edulis*, recovered from the intestine of a hamster seven days later, somewhat flattened, 0.43 mm. long, ventral view.

FIGURE 5. Mature but not fully gravid specimen, removed from the mantle of *M. edulis* and developed ten days in a recently hatched chick of *S. mollissima*, 0.72 mm. long, dorsal view.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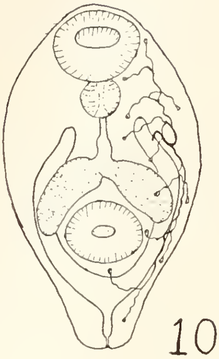
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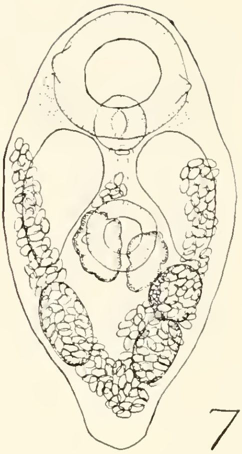
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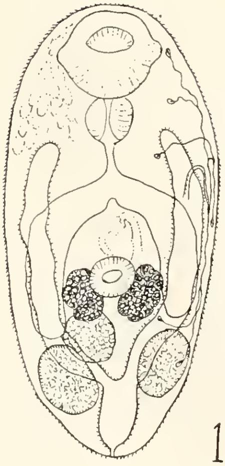
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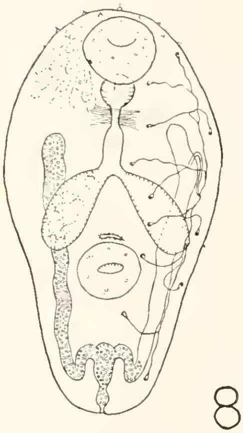
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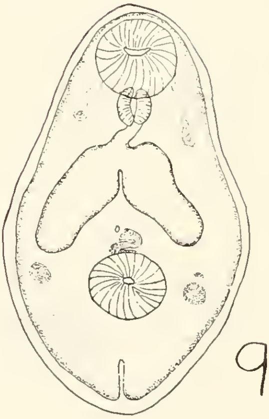
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PLATE II

FIGURE 6. Adult, flattened specimen from the intestine of *S. mollissima*, experimental infection, after feeding metacercariae from *Gemma gemma*. Specimen 0.24 mm. long, ventral view.

FIGURE 7. Another specimen, same infection as before, worm not flattened, 0.22 mm. long, ventral view.

FIGURE 8. Metacercaria from *Mya arenaria*, Woods Hole, Mass., 0.9 mm. long, excretory system added from sketches made when worm was alive.

FIGURE 9. Metacercaria from *Mytilus edulis*, Newburyport, Mass., same species as Figures 4 and 5. Specimen 0.46 mm. long.

other shore-birds members of the same or different species; (3) are the worms in the gall bladder, the intestine, the ceca, and bursa Fabricii of eider ducks members of the same or different species; (4) do the asexual generations of sporocysts in *Gemma gemma*, in *Hiatella arctica*, *Mytilus edulis*, and *Mya arenaria* belong to the same or different species; (5) which, if any, of the reported metacercariae and adults are later stages in the life-cycle of *Cercaria myae?* In an attempt to find answers to these questions the following procedures were devised: (1) collect and compare worms from the several locations in eider ducks and herring gulls (*Larus argentatus*); (2) determine whether the larvae (miracidia) in the eggs of these worms are mature and infective or whether the eggs need to be embryonated; (3) try to infect the different species of bivalve mollusks with eggs from worms taken from different hosts and different locations; (4) attempt transplantation of metacercariae from each species of bivalve to each of the others; (5) attempt infection of eider duck chicks with metacercariae from *G. gemma*, *H. arctica* and *M. arenaria*; (6) attempt infection of mammalian hosts with metacercariae from each of the molluscan hosts.

The material so far available[3] consists of adult worms from natural infections in the bursa Fabricii and gall bladder of *Somateria mollissima* taken at Boothbay Harbor, Maine and others from experimental infections of the intestine of eider chicks after feeding metacercariae from the mantle of *M. edulis* and from the mantle of *G. gemma*.

Metacercariae have been found on the mantle of *M. arenaria* from Boothbay Harbor, Maine and Woods Hole, Massachusetts; on the mantle of *Hiatella arctica* and *Gemma gemma* from Boothbay Harbor; and on the mantle of *Mytilus edulis* from Newburyport, Massachusetts and Milford, Connecticut.

Sporocysts and cercariae from *M. arenaria* were described by Uzmann (1952). Further details are given in the present paper. Sporocysts and cercariae from *H. arctica* and from *G. gemma* are described in this report.

Laboratory-hatched eider ducks, recently hatched herring gulls, golden hamsters, and white mice have been used as possible experimental hosts. Adult and recently hatched herring gulls were provided by the Marine Biological Laboratory.

EXPERIMENTAL RESULTS

Attempts to infect hamsters and white mice by feeding metacercariae from the three species of mollusks were not successful. Metacercariae from *Mya arenaria* and *Hiatella arctica*, although fed in large numbers, failed to infect eider ducks. As noted, metacercariae from *Mytilus edulis* and from *Gemma gemma* developed to sexual maturity in laboratory-raised and previously unexposed eider ducks. These worms were not only specifically distinct, but belong to different genera, as recounted in the descriptive section of the present paper.

[3] In a personal communication, Dr. John S. Rankin, Jr., reported that in 1938 he collected about one hundred gymnophallid trematodes from the ring-necked plover, *Charadrius semipalmatus*, which it is hoped may become available for study.

FIGURE 10. Metacercaria from *Hiatella arctica*, Boothbay Harbor, Maine. Specimen 0.21 mm. long, pressed to study the excretory system.

FIGURE 11. Metacercaria from *Gemma gemma*. Specimen 0.2 mm. long, same species as Figures 6 and 7.

Many worms were taken from the bursa Fabricii of eider ducks at Boothbay Harbor. Eggs of these parasites are embryonated when passed in the feces of their hosts, but do not hatch in sea-water. Accordingly, it is reasonably certain that the miracidia emerge only after the eggs reach the digestive tract of the first intermediate host. Eggs of worms, found as natural infections in the bursa Fabricii of eider ducks, were further embryonated and pipetted into the mantle cavities of *Mya arenaria* and *Mytilus edulis*. Other eggs were placed with specimens of *Hiatella arctica* in a gallon jar, half-filled with sea water and agitated by a stream of compressed air. The attempts to infect mollusks with eggs of the parasites were entirely fruitless. There is no assurance that the eggs were actually ingested although in natural infection it is probable that the eggs enter the mantle cavity with water currents, become enmeshed in the mucus that covers the gills, and reach the digestive tract in the stream of material that is driven by ciliary action from the gills to the mouth.

Metacercariae were removed from *M. arenaria*, *H. arctica*, and *G. gemma* and introduced into the mantle cavities of each of the other species. In no instance was successful transplantation assured. Even attempts to transfer metacercariae from one *M. arenaria* to another gave uncertain results. Often the worms failed to adhere to the new host. Also, the recipients may have been infected before the introduction of the new worms and a degree of resistance may have been developed. When experiments must be conducted on specimens that have been exposed previously to the same or related parasites, results must be subjected to rigid scrutiny.

DESCRIPTIVE RESULTS

Adult worms

I. From the bursa Fabricii of *Somateria mollissima* taken at Boothbay Harbor, Maine (Fig. 1)

About one hundred worms were collected; some were studied alive, others were fixed and stained for morphological study, and the others were dissected to obtain eggs for infection experiments. The worms are oval to pyriform, usually rounded anteriorly and more pointed posteriorly. When active the sides may be almost parallel and either end may be wider. They vary from 0.47 mm. long by 0.30 mm. wide to 1.00 mm. long by 0.50 mm. wide, the size of the somewhat flattened specimen shown in Figure 1. The cuticula is covered with scale-like spines, somewhat smaller posteriorly. The acetabulum is situated slightly behind the middle of the body and measures 0.10 to 0.14 mm. in diameter. The anterior portion of the body contains many gland-cells whose ducts open to the surface, chiefly around the oral sucker. The oral opening is subterminal, the sucker measures 0.13 to 0.18 mm. in diameter and the pharynx, which follows immediately, is 0.045 to 0.055 mm. in diameter. The esophagus varies much in length with the extension and retraction of the anterior portion of the body and measures 0.06 to 0.20 mm. in length. The ceca are relatively short and may, when the body is contracted, extend to the level of the acetabulum. The excretory vesicle is Y-shaped, with long arms which when filled may extend to the level of the pharynx. They follow the contour of the lateral edges of the body, but in living specimens the anterior ends may become widened, flattened, lobed or slightly bifid, and retraction of the vesicle along the lateral faces of the ceca may produce the lyre-shaped appearance figured in many species of *Gymnophallus*. The flame-cell pattern was not completely worked out, but insofar as could be determined, it is $2 [(2 + 2 + 2) + (2 + 2 + 2)]$, identical with that in the metacercariae from *M. arenaria* and *H. arctica* (Figs. 8, 10).

The testes are oval, and vary much in size in different individuals. They measure 0.06 to 0.13 mm. by 0.04 to 0.10 mm.; are lateral, acetabular to completely postacetabular, almost opposite; the one on the ovarian side is usually slightly more posteriad. Sperm ducts arise at the anterior ends, pass forward medially and dorsally and join to form the seminal vesicle which lies dorsal and anterior to the acetabulum. From a dorsal or ventral view it may appear ovoid or pyriform, but in lateral aspect the bipartite character is clearly apparent. The vesicle is followed by a cylindrical duct, enclosed in large prostate cells, which extends posteriad and ventrad opening into the small genital atrium. The common genital pore is median, just in front of the acetabulum. The ovary is lateral, usually on the left side, at the acetabular level. It is about the size of a testis and may partially overlap the testis of that side or the two may be separated, the ovary its own diameter in front of the testis. The oviduct arises at the median posterior aspect of the ovary, passes mediad and backward where it expands into a fertilization space from which Laurer's canal passes to the dorsal surface of the body. Immediately following, it receives the short vitelline duct and expands into the ootype, enclosed in the cells of Mehlis' gland. There is no seminal receptacle; instead, the initial section of the uterus is expanded and filled with spermatozoa. The course of the uterus is not constant; some of the loops described later may not be present, and the extent is determined in part at least by the number of eggs in the body. Typically, from the ootype the uterus passes backward, forms a loop or series of coils and then crosses behind the acetabulum to the antovarian side of the body. It then makes a backward loop, sometimes almost to the posterior end of the body, then a loop or series of coils forward almost to the level of the pharynx, then backward where behind the ceca it crosses to the ovarian side and coils may pass forward to the level of the pharynx, then backward mediad of the ovary, and then forward to the genital pore. The vitelline follicles are at the sides and above the acetabulum, six to twelve indistinct lobes on each side; they may extend through the ovarian and testicular zones; ducts from each side meet behind the acetabulum to form a small receptacle from which the common duct leads to the oviduct. The eggs are operculate, oval, and measure 0.021 to 0.025 mm. by 0.015 to 0.018 mm. (average 0.023 by 0.016 mm.).

II. From the gall bladder of *S. mollissima* taken at Boothbay Harbor, Maine (Figs. 2, 3)

Two specimens were found in the gall bladder of one bird; the bladders of seven other ducks were negative. The worms were about the same size; extended they measured 1.72 mm. long and 0.60 mm. in width and contracted 1.00 mm. long and 0.90 mm. in width. Although about twice as large as the worms from the bursa, these specimens were similar in shape and moved in a similar manner. However, as in most trematodes, the shape and relative position of structures are so pliable that an account based on a single specimen may be very misleading. Figures 2 and 3, made from the same specimen, show changes in shape when alive and moderately relaxed and when fixed under coverglass pressure with the anterior portion retracted and the posterior portion extended. Like the worms from the bursa, the anterior portion of the body contains many glandular cells. The acetabulum measures 0.17 to 0.185 mm. in diameter. The oral sucker is 0.18 to 0.23 mm. in diameter; the diameter of the pharynx is about one-third that of the oral sucker. The bifurcation of the digestive tract is about midway between the suckers and the ceca extend to the acetabular level. The excretory vesicle is almost identical with that of the worms from the bursa.

The testes are oval, 0.15 to 0.19 mm. in diameter, typically postacetabular; the seminal vesicle is in part dorsal to the acetabulum and the prostatic portion curves ventrad and posterad in front of the acetabulum to open into the genital atrium. The opening to the exterior is immediately anterior to the aperture of the sucker and may appear below the front portion of the acetabulum. The ovary is about the same size as the testes, lateral, sinistral in both specimens, at the acetabular level or slightly posteriad. The relations of the oviduct, ootype, and associated structures are quite similar to those in the worms from the bursa. The course of the uterus is similar also, with coils that extend almost to the posterior end of the body and others to the region of the pharynx. Some of the coils that initially were more median in position were pushed laterally by pressure of the coverglass. The vitelline follicles are lateral and posterior to the acetabulum; they extend through the ovarian and part of the testicular zone, but are in large part postacetabular. The eggs are about the same size as those in the worms from the bursa and average 0.023 by 0.017 mm.

III. From the intestine of *S. mollissima*; experimental infection after feeding metacercariae from *Mytilus edulis*, Long Island Sound, New York (Figs. 4, 5)

As noted earlier, sexually immature worms were recovered from the intestine of a hamster, seven days after feeding metacercariae from *M. edulis*. The largest worm, shown in Figure 4, is not quite sexually mature but measures, fixed and stained, 0.43 mm. long and 0.235 mm. wide. Feeding experiments were carried out later with eider chicks hatched in the laboratory from eggs sent from Boothbay Harbor, Maine. Feeding began when the birds were one day old. After ten successive days of feeding metacercariae, a series of worms was taken from the intestine of one bird. One worm, which had just begun egg-production, with seven eggs in the initial part of the uterus, is only slightly larger than the one from the hamster. The largest specimens, which measure 0.57 to 0.72 mm. in length, are not completely gravid, although coils of the uterus extend posteriad about one-half the distance from the acetabulum to the end of the body and forward to the pharynx. The worm which was just attaining maturity is 0.44 mm. long and 0.25 mm. wide. The acetabulum is 0.08 mm. in diameter; the oral sucker 0.092 mm. in diameter, and the pharynx is 0.057 mm. wide and 0.045 mm. long. The seminal vesicle is 0.056 mm. in diameter; the right testis is 0.09 by 0.06 mm., the left testis 0.09 by 0.056 mm.; the ovary 0.080 by 0.074 mm., and the eggs 0.027 by 0.020 mm. One of the largest specimens, shown in Figure 5, somewhat flattened, is 0.72 mm. long and 0.36 mm. wide. The acetabulum is 0.096 mm. in diameter; the oral sucker is 0.12 mm. wide and 0.10 mm. long; the pharynx is 0.057 mm. wide and 0.050 mm. long. The seminal vesicle measures 0.090 by 0.062 mm.; the right testis is 0.126 by 0.078 mm.; the left testis is 0.12 by 0.080 mm.; the ovary is 0.12 by 0.083 mm.; and the eggs average 0.026 by 0.019 mm. It appears that the first eggs are slightly larger than those produced later.

IV. From the intestine of *S. mollissima*; experimental infection after feeding metacercariae from *Gemma gemma* taken at Boothbay Harbor, Maine (Figs. 6, 7)

Laboratory-raised specimens of *S. mollissima*, never exposed previously to infection, were fed metacercariae on alternate days from October 13 to October 23, 1957, a total of five feedings. More than 100 worms, most of them sexually mature, were recovered from the intestine. Gravid specimens measured 0.15 to 0.33 mm. in length and 0.09 to 0.16 mm. in width. Juvenile worms were only slightly smaller. The cuticula bears flat, scale-like spines. The acetabulum is 0.029 to 0.037 mm. in diameter, only about one-half the size of the oral sucker. It is situated slightly posterior to the middle of the body. The oral sucker is 0.057 to 0.080 mm. in diameter; the posterior half of the sucker contains many unicellular glands which in living worms appear as yellowish columns, and the sucker when compressed has lateral ear-like projections (Figs. 6, 7). The pharynx is 0.027 to 0.033 mm. in diameter, almost as large as the acetabulum. The esophagus is variable in length; the ceca may be entirely preacetabular or approach the level of the gonads. The excretory vesicle is V-shaped with long arms which when filled may extend to the level of the pharynx. The flame-cell pattern could not be resolved since the worms were so filled with eggs that they recalled the figure of *Gymnophallus ovoplenus* as given by Jameson and Nicoll (1913). However, since the worms are almost fully grown in the metacercarial stage (Fig. 11), it is reasonably certain that the excretory system does not undergo change with reproductive maturity and the production of eggs. The testes are oval, 0.025 to 0.035 mm. by 0.035 to 0.050 mm. in diameter, situated on opposite sides in the posterior one-third to one-fourth of the body. Sperm ducts lead forward and mediad to open into a large, clavate seminal vesicle which extends from a level only slightly anterior to the testes almost to the bifurcation of the digestive tract. Anteriorly it becomes continuous with an ejaculatory duct, surrounded by secretory cells, which opens into the shallow genital atrium. The genital pore is median, some distance anterior to the acetabulum, often below the posterior edge of the pharynx. The ovary is lateral, either left or right, slightly smaller and immediately anterior to or overlapping the testis of the ovarian side. The oviduct arises at the median, posterior portion of the ovary, passing mediad where it enters the ootype. A seminal receptacle and Laurer's canal were not observed, but may be present. The vitellaria are composed of compact follicles, forming reniform glands, dorsal, lateral and somewhat posterior to the acetabulum. Vitelline ducts pass mediad and form a common duct which opens into the initial portion of the ootype. Mehlis' gland is present but consists of a small number of cells. The coils of the uterus may almost fill the body from the level of the oral sucker to the posterior end. The eggs measure 0.015 to 0.017 by 0.010 to 0.011 mm.

Metacercariae

I. From *Mya arenaria*, taken at Boothbay Harbor, Maine and at Woods Hole, Massachusetts (Fig. 8). Uniformly light infection, 1–12 worms per clam; about 25% of the clams infected

Specimens vary greatly in size as the unencysted metacercarial stage is an important growth phase in the life of the species. The worms actively ingest material from the mantle of the host and develop from a size hardly larger than a cercaria (0.12 to 0.25 mm. in length when retracted and extended) to almost definitive size (0.60 to 1.20 mm., corresponding measurements of length). Ordinarily the width is about one-half the length, but specimens may contract until the length and width are equal or elongate until the width is less than one-fourth the length. During the metacercarial period the larvae increase about five times in length and the organs, except the gonads and reproductive structures, attain almost full growth. There are six papillae around the acetabular opening. The acetabulum measures 0.13 to 0.14 mm. in diameter in the large individuals. The cuticular spines are pointed and sharp. The anterior end bears several small papillae, each tipped by a short, stiff bristle. The anterior region of the body contains a large number of unicellular glands which open around the oral sucker. The oral sucker is 0.147 to 0.16 mm. in diameter in large specimens. The digestive ceca are lined with large cells and yellow granules are conspicuous in the cytoplasm of the cells and in the lumen of the ceca. The yellow material appears like that in the digestive gland of the clam and may be taken by the parasite from the vascular fluid of the mollusk. The excretory system has been worked out and the arrangement of the tubules and flame-cells is shown in Figure 8. The formula is $2 [(2 + 2 + 2) + (2 + 2 + 2)]$; the common duct, which leads from the junction of the anterior and posterior collecting ducts, bears tufts of cilia which may simulate flame cells.

II. From *Mytilus edulis*, taken at Milford, Connecticut and Newburyport, Massachusetts (Fig. 9). Uniformly light infection, 1–10 worms per mussel; about 20% of the mollusks infected

The metacercariae in *M. edulis* produce lesions in the mantle and body-wall, which may result in the deposition of nacreous material. The larvae attain a length of 0.4 to 0.6 mm. and a width of 0.2 to 0.3 mm. In a fixed and stained specimen, 0.45 mm. long and 0.275 mm. wide, the acetabulum is 0.078 by 0.07 mm.; the oral sucker is 0.088 mm. and the pharynx is 0.030 mm. in diameter. Metacercariae from *M. edulis* developed to sexual maturity in a ten-day-old eider duck (*cf.* Figs. 4, 5).

III. From the mantle of *Hiatella arctica*, taken at Boothbay Harbor, Maine (Fig. 10). Uniformly light infection, 10 to 8 worms per clam; about 20% of mollusks infected

The metacercariae in *H. arctica* are similar in shape to those in *Mya arenaria*; the smallest individuals of the two species are about the same size, 0.12 to 0.175 mm. in length, but the largest worms from *H. arctica* do not exceed 0.3 mm. in length and accordingly are only about one-fourth as large as those from *M. arenaria*. The suckers, however, are relatively larger; the acetabulum measures 0.050 to 0.058 mm. in diameter in large specimens. The anterior end of the body does not contain the large number of glandular cells so characteristic of the worms from *M. arenaria* and the digestive ceca do not contain the yellow material so conspicuous in that species. The oral sucker of large individuals measures 0.054 to 0.064 mm. in diameter. Some of the measurements were made while the worms were under considerable pressure from the coverglass while the details of the excretory system were studied. The excretory system is identical with that in the larva from *M. arenaria* and the flame-cell formula is $2\,[(2+2+2)+(2+2+2)]$.

IV. From the mantle of *Gemma gemma* at Boothbay Harbor, Maine (Fig. 11). Uniformly light infection; 1 to 10 worms per clam; about 10% of the clams infected

These metacercariae were fed to an eider duck and became mature (*cf.* Figs. 6, 7). Specimens from *G. gemma* measure 0.13 to 0.23 mm. in length and 0.06 to 0.12 mm. in width. The anterior end contains many glandular cells that open around the oral sucker. The cuticula bears flat, scale-like spines. The acetabulum is just posterior to the middle of the body and is 0.028 to 0.033 mm. in diameter. The oral sucker measures 0.05 to 0.07 mm. in diameter in large individuals. The posterior half of the sucker contains yellowish columns as described for the adult and when compressed, the sucker has lateral ear-like projections. The pharynx is 0.026 to 0.030 mm. in diameter and the ceca usually extend into the postacetabular zone and often to the gonads. The reproductive structures are well developed. The testes, ovary and vitelline glands are almost as large as in the sexually mature individuals and the terminal portions of the ejaculatory duct and metraterm are shown in Figure 11. The excretory vesicle is typical of the gymnophallids but the flame-cell pattern is simpler; it is shown in Figure 11 and the formula is $2\,[(2+2)+(2)]$. Such a difference probably has considerable taxonomic significance.

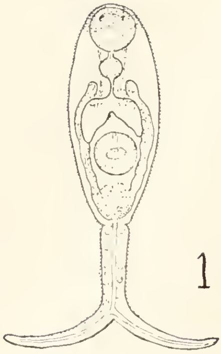
Sporocysts and cercariae

I. From *Mya arenaria* taken at Newburyport, Massachusetts and Boothbay Harbor, Maine (Fig. 12)

This species, described by Uzmann (1952) from *M. arenaria* taken at Newburyport, Massachusetts, has been found in the same host-species from Boothbay Harbor, Maine. The incidence of infection is low also in Maine; only three infections have been observed in the dissection of over 1000 clams. No cercariae were found in water in which the clams were kept, perhaps because the larvae were carried away in the current of water flowing over the mollusks. The cercariae were described and figured by Uzmann who noted that a similar and probably identical larva had been described by Stafford (1912). Uzmann also noted the close morphological agreement between the cercaria from *M. arenaria* and *Cercaria discursata* Sinitsin, 1911. Allison (1943) assigned *Leucochloridiomorpha constantiae* (Mueller, 1935), which has a furcocercous cercaria, to the family Brachylaemidae and suggested that *C. discursata* may belong to the same family. It now appears that the resemblance between the cercaria of *L. constantiae* and *Cercaria discursata* is merely superficial and that both *C. discursata* and *C. myae* should be placed in the subfamily Gymnophallinae.

II. From *Hiatella arctica* taken at Boothbay Harbor, Maine (Figs. 13, 14)

The description of this species is based entirely on material collected by Uzmann in 1953. Of 13 specimens examined on March 18th, 2 were infected; of 136 ex-



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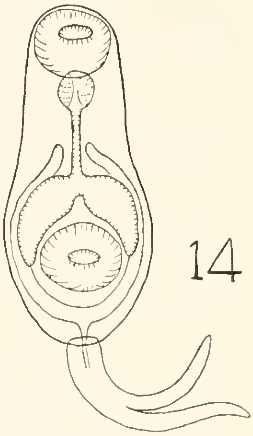

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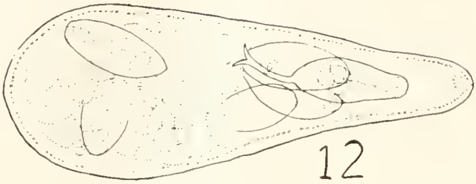

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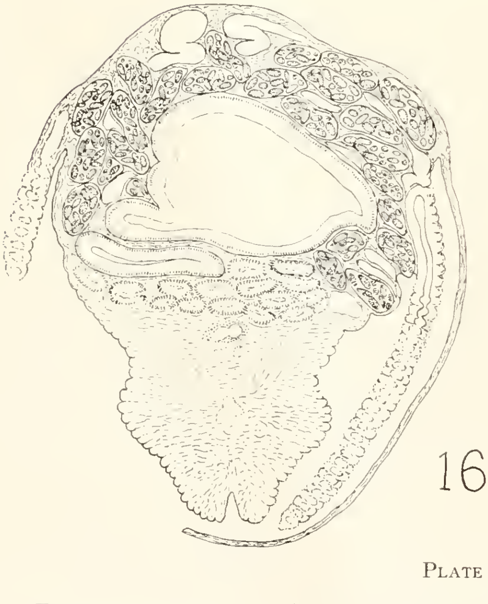

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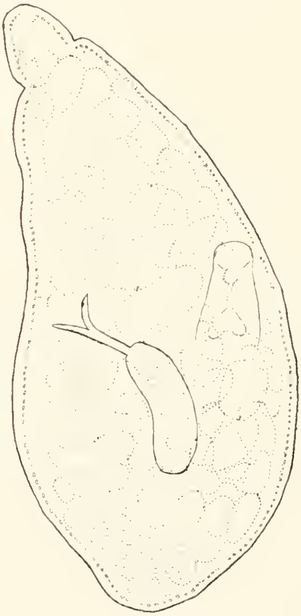

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PLATE III

FIGURE 12. Sporocyst, 0.67 mm. long, and cercariae from *Mya arenaria*. This is *Cercaria myae* Uzmann, 1952.

FIGURE 13. Sporocyst, 0.80 mm. long, and cercariae from *Hiatella arctica*. This species is identified as *Cercaria reesi* Hutton, 1953.

amined May 12th, 6 were infected. Several hundred specimens of *H. arctica*, taken from the same area where Uzmann made his collections, have been examined in the summers of 1956 and 1957 without finding the parasite.

The sporocysts are oval to pyriform, usually with a neck-like extension which may be one-fourth or even one-third of the total length of the sporocyst and which bears the birth-pore at the end. The largest fixed and stained sporocyst is 0.96 mm. long and 0.37 mm. wide, but most of them are much smaller. The one shown in Figure 13 is 0.8 mm. long. The cercariae have forked tails, spined cuticula and no eye-spots; the body is 0.12 to 0.175 mm. in length and 0.05 to 0.068 mm. wide. The acetabulum is 0.036 to 0.043 mm. in diameter and the oral sucker is approximately the same size. The tail-stem is variable in length, 0.02 to 0.052 mm., and the furci are somewhat longer when fully extended, but they may contract to less than one-half the fully extended length. The pharynx is 0.015 to 0.022 mm. in diameter, spherical to oval, and longer than broad when the anterior end is protruded. The cephalic glands and ducts could not be resolved with certainty. The excretory vesicle is shown in Figure 14, but since the supply of living material was limited, the flame-cell formula was not worked out.

III. From *Gemma gemma*, taken at Boothbay Harbor, Maine (Figs. 15, 16)

No infection by this species was discovered by isolation of the clams, perhaps because the larvae swim well and were carried away in the running water that bathed the mollusks. Of 824 *G. gemma* dissected, three were infected. The sporocysts were dispersed in the interlobular spaces of the digestive gland. They are cylindrical to oval to pyriform to clavate, with a narrow end in which the birth-pore is situated. The smaller ones are motile while the larger ones lose motility as they become filled with cercariae. They may extend to a length of about 0.50 mm. The cercariae emerge from the clam and swim vigorously. In swimming, the tail is turned ventrad, the furci are extended and lash from side to side while the anterior end wobbles back and forth. Otherwise, the tail manifests nervous twitching movements while the furci separate and then come together. When killed by adding hot A F A (alcohol-formol-acetic acid) solution to a beaker containing a small amount of swirling sea-water in which they are suspended, the cercariae are very uniform in size and shape (Fig. 15). The cuticula bears spines on both the body and tail. The body is 0.12 to 0.14 mm. in length and 0.04 to 0.05 mm. in width. The tail-stem is 0.04 to 0.05 mm. in length, 0.015 to 0.017 mm. wide at the base and 0.013 to 0.015 mm. wide at the bifurcation of the tail. The furci are 0.060 to 0.068 mm. long and 0.011 mm. wide at the base. Alive, the cercarial body varies from 0.08 to 0.2 mm. in length and 0.03 to 0.07 mm. in width; the tail varies from 0.05 to 0.14 mm. in length, the stem from 0.025 to 0.06 mm. and the furci from 0.025 to 0.13 mm. There are no eye-spots. The acetabulum, situated just posterior to the middle of the body protrudes slightly and measures 0.027 to 0.030 mm. in diameter. The oral sucker is the same size as the acetabulum; there is a short prepharynx, and the pharynx measures 0.014 to 0.019 mm. in

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FIGURE 14. *Cercaria reesi* Hutton, 1953 from *Hiatella arctica*, Boothbay Harbor, Maine.

FIGURE 15. Cercaria from *Gemma gemma*, Boothbay Harbor, Maine.

FIGURE 16. Cross-section of *G. gemma* through the pedal ganglion, mantle and part of the gill of one side removed, to show location and extent of the infection. At this level, the sporocysts are more numerous than the follicles of the digestive gland.

diameter. The esophagus varies in length as the body elongates and contracts; in fixed specimens it is about as long as the diameter of the pharynx. The ceca are ovate, wider anteriorly. They may be entirely preacetabular or extend posteriad to the level of the middle of the acetabulum; their walls are composed of large cells. A pair of lobed unicellular glands is situated, one on either side, at the level of the intestinal bifurcation and their ducts pass forward to open above the mouth. The excretory vesicle consists of a dorsal, pouch-like expansion from which, on the ventral side, the arms extend forward to the pharyngeal level. They are ventral to the digestive ceca and are filled with concretions, 0.005 to 0.006 mm. in diameter. On each side there is a flame-cell at the level of the pharynx, and the capillary from it divides at the level of the intestinal bifurcation; one branch leads to the vesicle but the other could not be followed with certainty. It may extend back to a flame-cell located at the level of the vesicular pouch, but the duct from that cell was hidden at the acetabular level by concretions in the excretory vesicle.

Discussion

The present account includes descriptions of sporocysts, cercariae, and metacercariae from marine bivalves and adult worms from the eider duck, *Somateria mollissima*. Specific identification is so uncertain that we prefer to list the worms by host and location rather than propose names that might further confuse the taxonomic situation.

The adult forms I, II, and III are members of the genus *Gymnophallus*. Worms from the bursa Fabricii of *S. mollissima*, Adult No. I, although they are somewhat smaller, may be identical with *G. bursicola* Odhner, 1900 or *G. dapsilis* Nicoll, 1907, if indeed these species are actually distinct. It is interesting to note that Jameson and Nicoll (1913) reported *G. dapsilis* from the intestine as well as the bursa. Our Adult No. II, from the gall bladder, may be identical with *G. deliciosus* (Olsson, 1893) or *G. choledochus* Odhner, 1900, if the latter of these species is really valid. The account of Isaitchikow (1924), if it dealt with a single species and if that species was *G. choledochus*, would suggest that the reported differences between *G. choledochus* and *G. deliciosus* are not significant. The single specimen from the gall bladder of *S. mollissima* taken in Iceland and described as a new species, *G. bilis*, by Brinkmann (1956) is similar to *G. bursicola* and, despite reported differences, may belong to that species. The naming of a new species on a single specimen is not recommended. Our Adult No. III, from the intestine of an eider chick, the sexually mature stage of the metacercaria in *Mytilus edulis*, is larger than *G. somateriae* (Levinsen, 1881) and may be *G. bussicola*, which had not yet settled in the bursa and reached full size.

Our Adult No. IV is clearly a member of the genus *Parvatrema* Cable, 1953. The worms are very similar to *Parvatrema borinqueñae* Cable, 1953, the morphological differences are minor, the chief differences are in geographical location and in primary and secondary hosts. The cercariae from *Gemma purpurea*, described by Cable as larvae of *P. borinqueñae*, are smaller, the tail is relatively much smaller and the cercaria, according to Cable, "is a poor swimmer" when compared with the cercariae from *Gemma gemma* at Boothbay Harbor, Maine. Because of these differences, we recognize the worms we have described as a new and distinct species for which we propose the name *Parvatrema borealis*.[4] Cable noted the possible identity of *Parvatrema* and *Gymnophalloides.* Both Yamaguti (1939) and Cable (1953) accredit this genus to Fujita (1925) although it appears from the footnotes in his French translation of the text and from his accompanying note, that Dollfus claims credit for the generic name. The worms described as *Gymnophallus ovoplenus* by Jameson and Nicoll (1913) differ distinctly from *Gymnophallus* and although the morphology is very imperfectly known, it appears from the figure and account of Jameson and Nicoll that the worms agree better with the characteristics of *Parvatrema* and accordingly we transfer the species to the latter genus as *Parvatrema ovoplenus* (Jameson and Nicoll, 1913).

Metacercaria I is common in *Mya arenaria* but it did not persist or develop in mice, hamsters, eider ducks or herring gulls, and other stages in the life-cycle are yet unknown. Only when the cercarial and adult stages become available will the taxonomic position of the species be clarified.

Metacercaria II is common in *Mytilus edulis*; it developed in the eider chick and as noted, may be the asexual stage of *G. bursicola.*

Metacercaria III is relatively common in *Hiatella arctica* but like Metacercaria I, it did not persist or develop in experimental animals and its status is yet uncertain.

Metacercaria IV, from *Gemma gemma,* is the asexual stage of *Parvatrema borealis* n. sp., which developed in large numbers in the intestine of *S. mollissima.* Whether other birds also serve as final hosts is unknown but probable.

The sporocysts and Cercaria I, which may cause the condition known by clam diggers as "waterbelly," were described by Uzmann (1952). Although the species has received further study, later stages in the life-cycle of the parasite are still unresolved.

The sporocysts and Cercaria II, from *Hiatella arctica* at Boothbay Harbor, agree in detail with the corresponding stages of *Cercaria reesi* as described by Hutton (1953) from *H. arctica* and *Hiatella striata* at Plymouth, England. They are referred to that species.

The sporocysts and cercariae III, from *G. gemma* at Boothbay Harbor may be identical with the metacercariae from the same host-species which developed to maturity in *S. mollissima* and which we describe as *Parvatrema borealis* n. sp. If so, the oral sucker must double in diameter while the acetabulum remains essentially unchanged.

Present literature concerning gymnophalline trematodes discloses descriptions of twelve species of adult worms from the gall bladder, intestine, ceca and bursa Fabricii of various shore-birds; an even larger number of metacercariae from marine mollusks; and a score of different sporocysts and cercariae have been recorded from various marine bivalves. Yet there is no agreement on the number of valid species and specific relations between particular cercariae, metacercariae and sexually mature worms remain undetermined. As yet there are no precise data concerning host specificity, *i.e.,* the ability of one species to infect different primary, secondary, and definitive hosts. Furthermore, information concerning organ specificity is equally meager, and it is uncertain whether or not members of a given species can persist in more than one location in or on a given host. Comparison of specimens from different hosts and different locations does not provide satisfactory answers;

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[4] Holotype and paratype deposited in U. S. Nat. Mus., Helminth. Coll. No. 56235.

the selected site in each instance may be determined by physiological adjustments. The extent of morphological variation that may result from development in different hosts or different locations is quite unknown. Only when life-cycles have been discovered and controlled experiments permit tests on host and organ specificity, will it be possible to determine the extent of variation in individual species and the validity of the several named adult and larval forms.

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THE REPETITION OF PATTERN IN THE RESPIRATION OF UCA PUGNAX

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Persistent rhythms of O_2-consumption for two species of fiddler crabs, *Uca pugnax* and *Uca pugilator*, were described by Brown, Bennett and Webb in 1954. Analyses of the data revealed rhythms of several different periods including diurnal, semi-lunar and lunar ones. Of particular interest, from the point of view of the mechanism by which biological rhythms are maintained, is the observation that two rhythms of such similar periods as 24.0 hours (diurnal) and 24.8 hours (lunar-day) persist in an organism under constant conditions. The present investigation has been carried out in an attempt to characterize these two rhythms in terms of the regularity of period and of form, and to investigate the persistence of the lunar-day rhythm under conditions in which the ordinary tidal effects were absent from the environment.

MATERIALS AND METHODS

All of the animals used in these experiments were specimens of *Uca pugnax* collected at Chappoquoit Beach, Cape Cod, Mass. The animals were transported to the Marine Biological Laboratory in open containers and were kept there in enamel pans with a small amount of sea water.

O_2-consumption was measured by means of Brown respirometers (Brown, 1954), modified as described by Brown (1957). Four respirometer vessels were attached to a recording unit and the whole assembly placed in a sealed barostat. The barostat was evacuated to a pressure of 28.5 inches of mercury, which was somewhat below the expected minimum barometric pressure. A maximum of six such units was in operation at any one time. The barostats were opened at approximately three-day intervals at which time the ammonia and CO_2 absorbents were changed, the oxygen supply was replenished, and fresh animals were placed in the vessels. The barostats themselves were contained in water baths kept at a constant temperature of 24° C. and they were located in a room without windows and provided with constant illumination such that the illumination within the barostats was less than one foot-candle. The lever system of the recording units was such that the recording arms were displaced 1.2 mm. for each gram of weight increase of the respirometer vessels. The ink-writing pen of the recorder traced on millimeter graph paper which was marked off in hours after being removed from the drum. The displacement values for each hour were then recorded.

The methods by which the data so obtained were analyzed are described in the following section.

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[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, NONR-122803.

RESULTS AND ANALYSES

Hourly rates of oxygen consumption, when calculated as mean values for single days, reveal a range for the summer of 1957 of from 28 to 69 ml./kg./hr. The mean rate of oxygen consumption for the first lunar period of 1956 was found to be 32.4 ± 8.4 ml./kg./hr.; for the second lunar period it was 36.0 ± 6.9 ml./kg./hr. In the summer of 1957 the comparable values were 41.9 ± 9.4 ml./kg./hr. and 44.6 ± 11.9 ml./kg./hr.

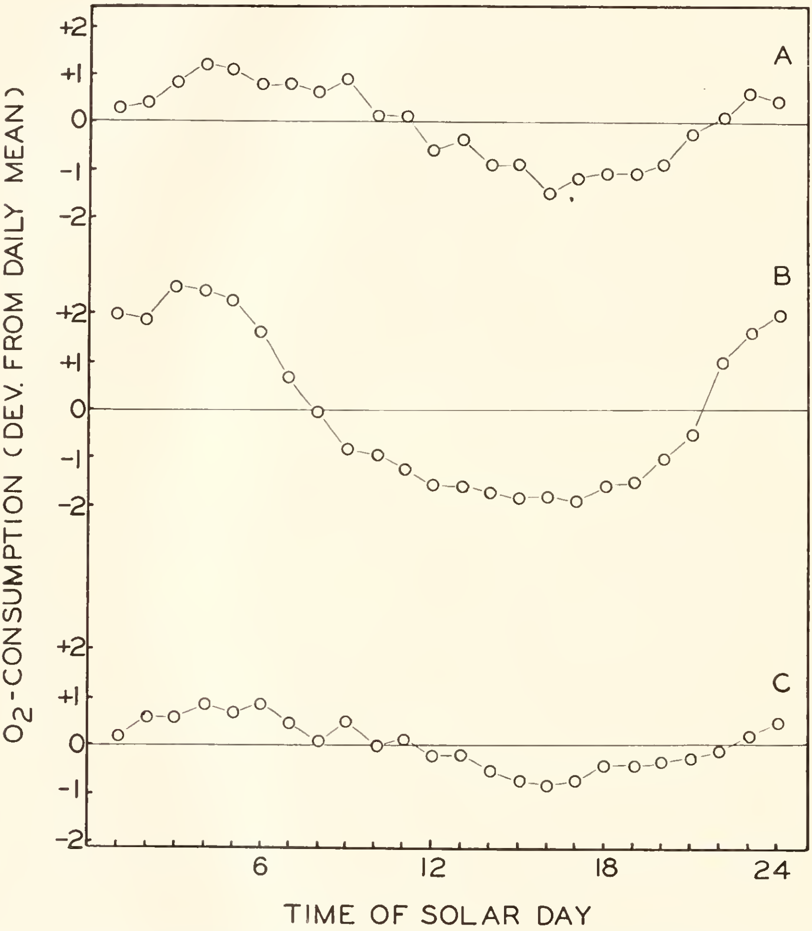

FIGURE 1. The mean diurnal variation in O_2-consumption of *Uca pugnax* for three 29-day periods, (A) July 15 to Aug. 13, 1955, (B) July 13 to Aug. 11, 1956, and (C) July 14 to Aug. 12, 1957.

In Figure 1 are seen the average diurnal curves for representative periods in three successive years. Each curve represents the mean hourly values (expressed as deviations from the mean) for a period of 29 days. Figure 1A represents data from two recording units for the period July 15 to August 13, 1955. Figure 1B shows similar values from five recorders for the period July 13 to August 11, 1956. Figure 1C shows the mean daily curve for the period July 14 to August 12, 1957. In this year six recorders were used with a minimum of two on any one day.

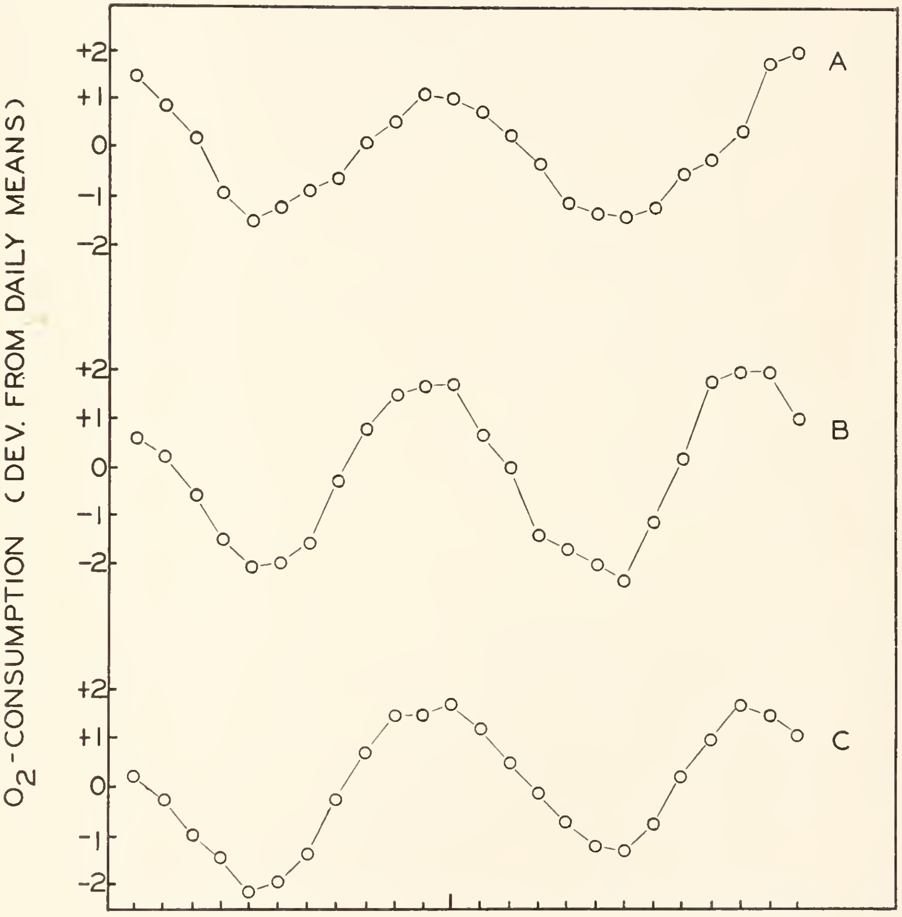

FIGURE 2. The primary lunar rhythm in O_2-consumption of *Uca pugnax* for three 29-day periods. (A) July 15 to Aug. 13, 1955, (B) July 13 to Aug. 11, 1956, and (C) July 14 to Aug. 12, 1957.

In all three cases the diurnal curve is characterized by a single maximum between 2 AM and 6 AM, then a broad minimum extending from about noon to 7 PM, after which there is an increase in rate that continues until midnight. In 1956 (Fig. 1B) the amplitude is greater than in the other two years, but the form and phase relations appear to be essentially the same in all three. The amplitude of the mean diurnal rhythm can be described by the ratio of maximum to minimum value. Expressed in these terms, the mean amplitude obtained in 1956 was 1.4, while that for 1957 was 1.2.

In Figure 2 are shown the primary lunar curves for the periods represented in Figure 1. The curves in Figure 2 were obtained by rendering random the diurnal variations while events of a lunar frequency were kept constant (see Brown, Bennett and Webb, 1954). The points are plotted in such a way that lunar zenith is at 12 hours and lunar nadir is at 24 hours. Figure 2A represents the data from 1955, Figure 2B those from 1956, and Figure 2C those from 1957. The similarity among the primary lunar curves for these three years is even more striking than that exhibited by the diurnal curves, since the likeness now includes amplitude as well as form and phase relationships. The ratio of maximum to minimum for the lunar rhythm remains at about 1.4 in all three years.

All of the curves in Figure 2 show two maxima and two minima. The peak rates of oxygen consumption are seen to occur at approximately lunar zenith and lunar nadir. Both maxima are about the same height and there is similarly little difference between the two minima in a lunar day. Since there appears to be little difference between events occurring at the time of lunar zenith and those at lunar nadir the effect is of a rhythm with a period of about 12 hours. Further, since the amplitude of the lunar rhythm is at least as great as that of the diurnal one (and for 1957 it is considerably greater), one would expect that the curves for respiration on single days would exhibit the lunar component rather prominently and that the form of the daily curves would tend to repeat at approximately 15-day intervals.

A direct and elementary test for 15-day repetition of form is possible from the data presented in Figure 3. In this figure each point represents the average of all machines recording on the particular day. The ordinate values are the displacement in mm. of the recording levers. The number of measurements contributing to each point ranges from three to six. All of the data are from the summer of 1957 and the days represented are as follows: Curve A, for June 24, is the third day before new moon; Curve B, for July 2, the fifth day after new moon; Curve C, July 8, the third day before full moon; Curve D, July 16, the fifth day after full moon; Curve E, July 23, the third day before new moon; Curve F, July 31, the fifth day after new moon; Curve G, August 7, the third day before full moon; Curve H, August 15, the fifth day after full moon; Curve I, August 22, the third day before new moon; and Curve J, August 30, the fifth day after new moon. Thus, reading across the figure, Curves A, E, and I are synchronous with respect to lunar period; each represents the third day before new moon. Curves B, F, and J are synchronous, each representing the fifth day after new moon. Curves C and G both represent the third day before full moon, while Curves D and H represent the fifth day after full moon. If one takes a semi-lunar rather than a lunar period, then alternate curves throughout the figure are synchronous. Thus, Curves A, C, E, G, and I are effectively synchronous in semi-lunar periods.

Examination of Figure 3 shows that on each of the days represented there are fluctuations such that maximal values represent two to three times the minimal values for the day. Two maxima and two minima occur daily, dividing the day roughly into quarters. It is also obvious that the curves can readily be divided into two classes: those which exhibit a maximum in the hours between 6 AM and 12 N and a minimum between 12 N and 6 PM, and those which show a minimum between 6 AM and 12 N and a maximum between 12 N and 6 PM. Curves A, C, E, G, and I fall into the first category and all of the others into the second. In the period immediately preceding new moon (*e.g.*, curve A) lunar zenith occurs in the late morning hours, while as full moon approaches (*e.g.*, curve C) lunar zenith will be in the late evening hours. The data presented in Figure 3 give no evidence that there is any consistent difference between a semi-lunar period including new moon and one including full moon. The relative heights of respiratory maxima on any day seem not to be greatly affected by the time of day at which lunar zenith occurs. These data support the description derived from the mean lunar day curves. The times of lunar zenith and lunar nadir are clearly indicated in the respiratory data for single days, and these times are indicated by major maxima. The respiratory pattern is thus repeated at approximately 15-day intervals.

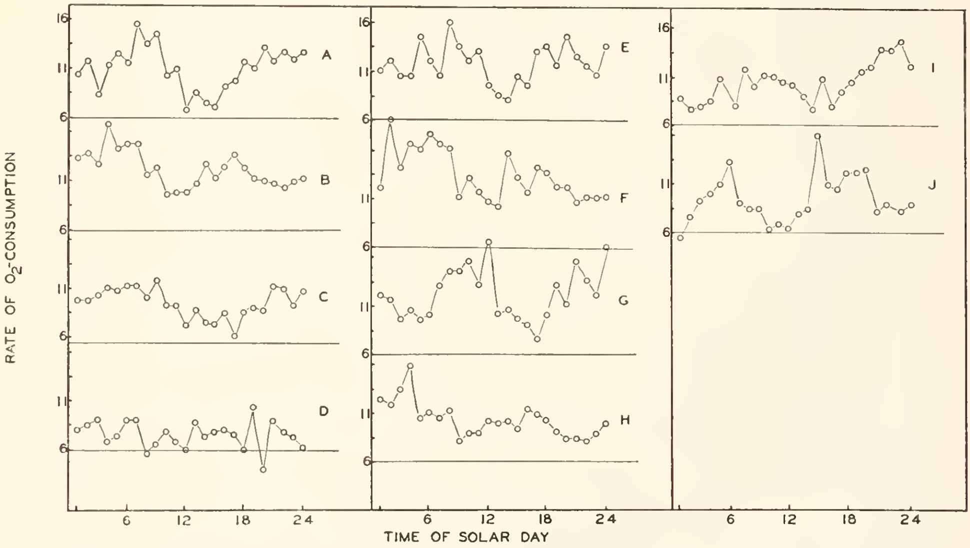

FIGURE 3. Variation in O_2-consumption of *Uca pugnax* on single days during the summer of 1957. See text for further explanation.

Although the major maxima and minima are, in general, readily distinguished in Figure 3, it is seen that on many days single points appear which deviate widely from the trend of the series of points around them. Such points may make difficult the comparison of the form of curves, especially on days on which the amplitude of fluctuation is low. Another aspect of the data that might contribute to the difficulty of analysis of form is the day-to-day variation in the level at which O_2-consumption occurs. Such variation might permit a single day with high values to contribute disproportionately to the form of a mean curve for periods of several days taken together. The first difficulty can be minimized by the use of successive, overlapping three-hour averages to obtain the hourly values. The effect of variation in level of O_2-consumption can be reduced by using the ratio of hourly values to mean value for the day.

Evidence that the use of three-hour overlapping averages preserves the form and phase relations of fluctuations occurring over a period of four or more hours, while eliminating the irregularities of single hours, is presented in Figure 4. In this figure are plotted (A) the primary lunar curve for the period July 14 to August 12 obtained by converting the raw hourly data to the ratio of hourly value to mean for the day and then rendering random the diurnal variations in the manner previously described. Curve B, Figure 4, shows the primary lunar curve obtained by use of overlapping three-hour averages for hourly values. The data are for the same period as those in Curve A. (Figure 4B is the same curve as was seen in Figure 2C.)

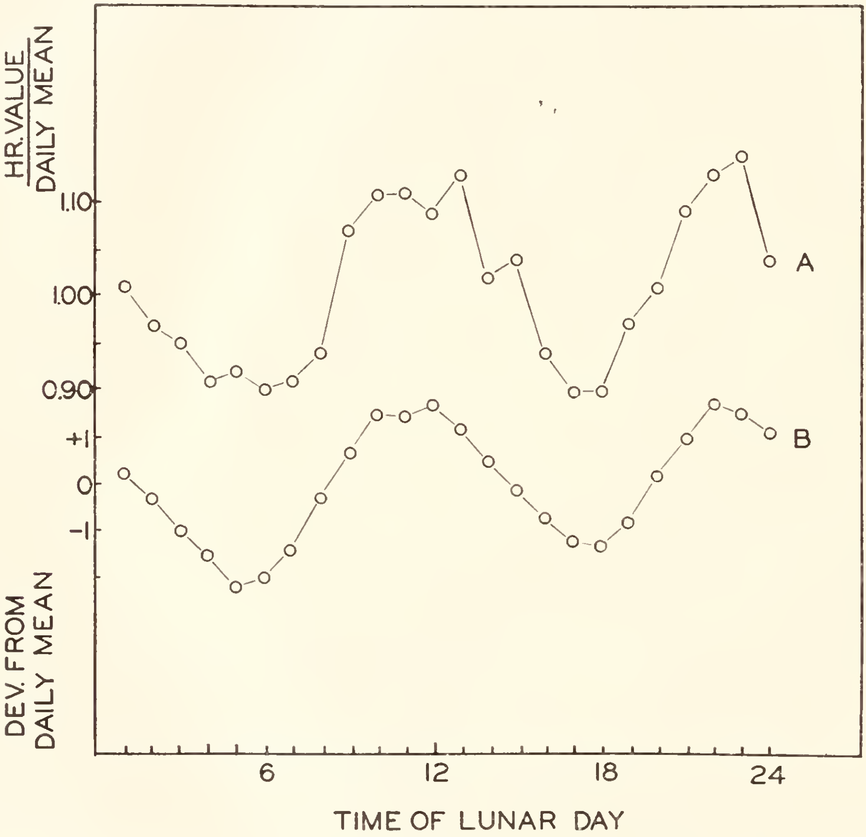

FIGURE 4. The primary lunar rhythm in O_2-consumption of *Uca pugnax*. Curve A presents raw data, Curve B presents data smoothed by use of overlapping 3-hour averages.

As might reasonably have been expected, Curve B is smoother than Curve A—that is, there is some loss of sharpness of definition so far as points of transition are concerned. It is quite clear, however, that if one is concerned with the form

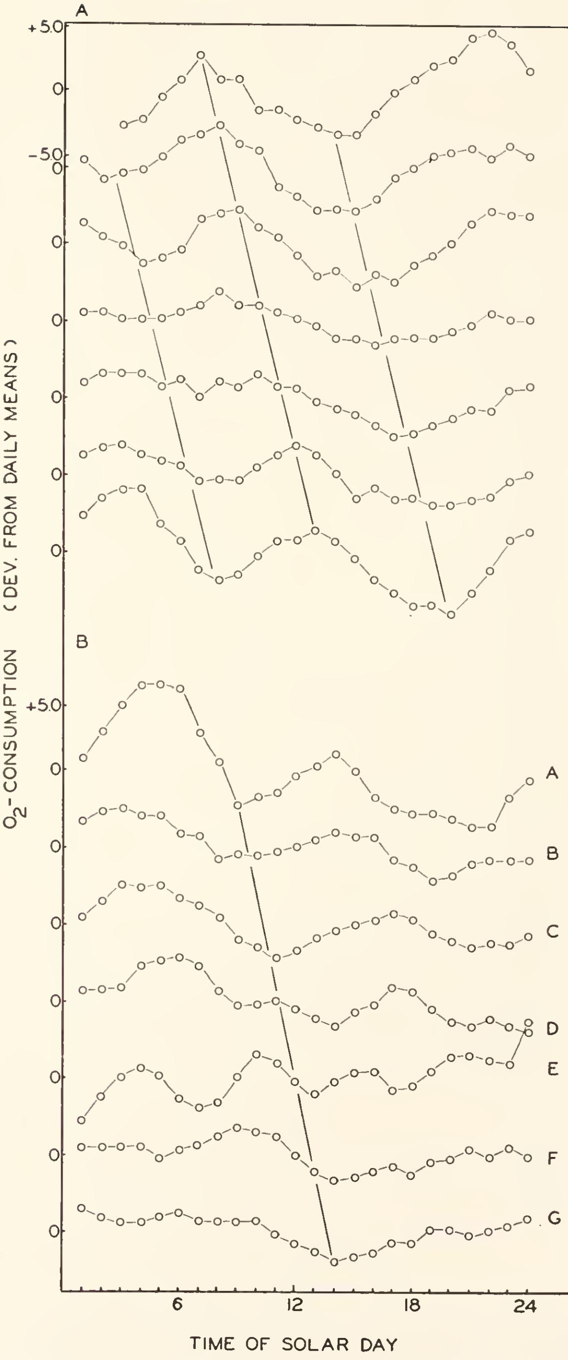

FIGURE 5. Variations in O_2-consumption of *Uca pugnax* for two successive 7-day periods, (A) June 23 to June 29, and (B) June 30 to July 6, 1957. The ordinate scale used throughout the figure is that indicated for the top curves.

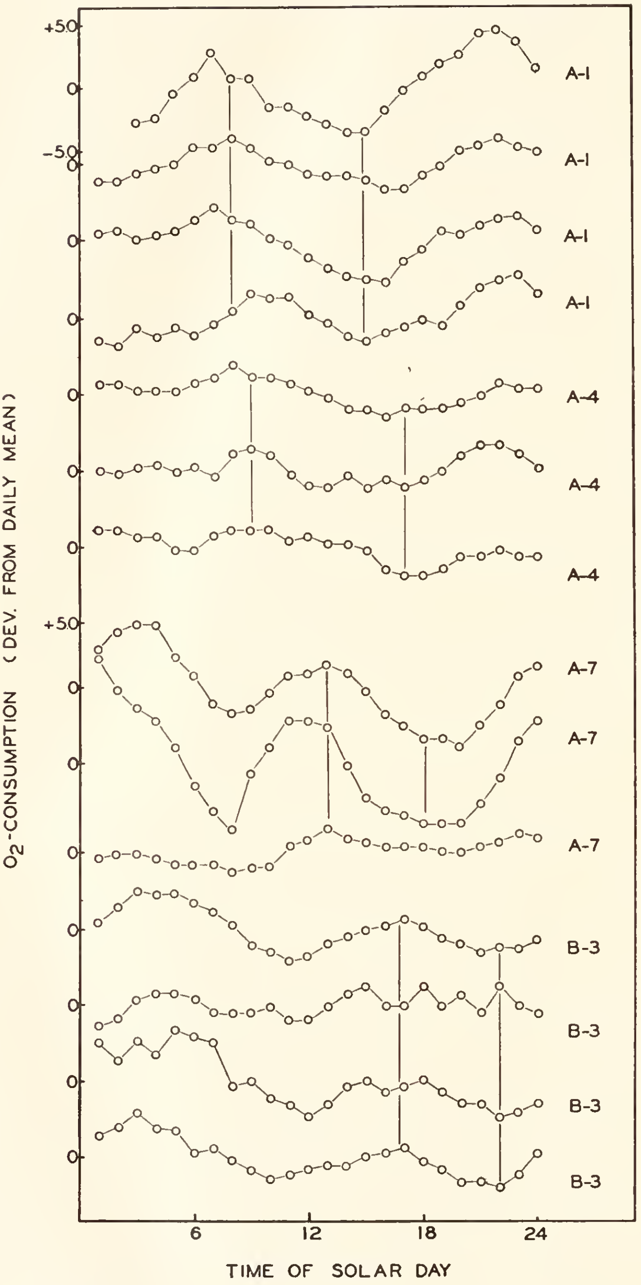

FIGURE 6. Variations in pattern of O_2-consumption throughout semi-lunar period as shown by the recurrence of form on comparable days. The ordinate scale used throughout the figure is that indicated for the top curve.

and placement of major fluctuations occurring over periods of several hours Curve B is adequate. If one is concerned with the precise difference between adjacent hours Curve A would be preferable. Since we are in the present work interested only in fluctuations with periods of 12 to 24 hours we shall use overlapping three-hour means in the analyses of the data.

The day-to-day changes in pattern associated with the overt lunar rhythm are illustrated in Figure 5 which shows hourly data for successive single days. Each point represents the mean hourly value for all recorders operating on that day, the values being expressed as deviations from the mean for that day.

In Figure 5A are seen the O_2-consumption data for the seven days beginning June 23, 1957 (top curve) and ending June 29, 1957 (lowest curve). The diagonal lines indicate progression of maxima and minima across the day. It can be seen that both maximum and minimum have advanced 6 hours in the 7 days illustrated. This is a rate of 51.4 minutes per day. This is to be compared with about 50 minutes per day, the rate of lunar progression. In the period represented, new moon occurred on the fifth day.

In Figure 5B are seen data for the seven days immediately following those in 5A, *i.e.*, June 30 to July 6 inclusive. It is seen that only the first four of these curves are clearly bimodal. Moreover in these first four there does not appear the clear progression of a peak through the day that was observed in the preceding seven days. In Curve A, Figure 5B, two maxima are obvious, one at about 5 AM and the second at 2 PM. Both maxima seem to have disappeared in the last three curves of the series. The minimum present in Curve A can be identified with one present in each of the last three, as is indicated by the diagonal line. The rate of progression of this minimum is such that in 7 days it has moved 5 hours, or about 43 minutes per day. In the two 7-day periods represented in Figure 5, a single minimum has progressed 11 hours in 14 days, at the over-all rate of 47.2 minutes per day, which is quite good agreement with the average rate of lunar progression.

To demonstrate the recurrence of characteristic forms of the daily pattern of respiration at comparable times in a semi-lunar period, Figure 6 has been prepared. For convenience of description and labelling a lunar period is divided into units of 7 or 8 days each as follows: June 23 to June 29 is called an A period, consists of 7 days of which the fifth is the day of new moon. The period from June 30 through July 6 is a B period and is 7 days long. From July 7 to 13 inclusive is again an A period, is 7 days long, and full moon occurs on the fifth day. Continuing through the summer in the same manner, A periods are always 7 days long and new or full moon occurs on the fifth day. The intervening days are included in the B periods which may be either 7 or 8 days, depending on the number of days available. In this way any given day of a semi-lunar period can be identified by a letter and a number. The days so represented in Figure 6 are: A-1, A-4, A-7, and B-3. Each group of days synchronous with respect to semi-lunar period is indicated by two vertical lines connecting members of a group at maxima and minima.

The first group of curves in Figure 6, representing four A-1 days during the summer of 1957, shows quite clearly the resemblance among A-1 days even though separated in time by as much as two months. All of the other groups also reveal great internal similarity. Moreover, any member of A-1 is more nearly like any member of A-4 than like any member of A-7 or B-3, regardless of the absolute

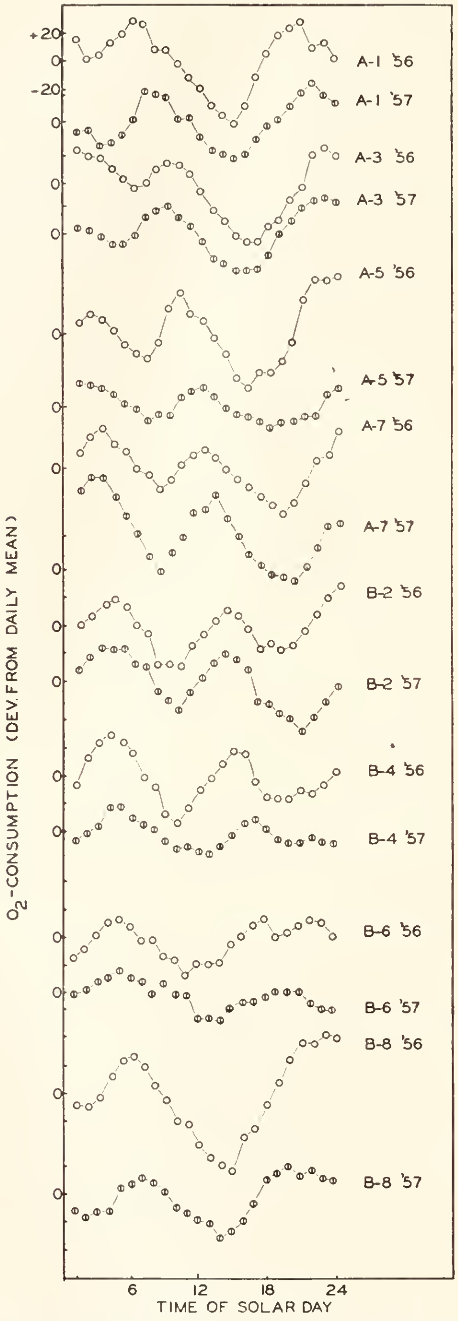

FIGURE 7. Comparison of changes in pattern of respiration throughout mean semi-lunar period as obtained in 1956 (upper member of each pair) with those obtained in 1957 (lower member of each pair). The ordinate scale used throughout the figure is that indicated for the top curve.

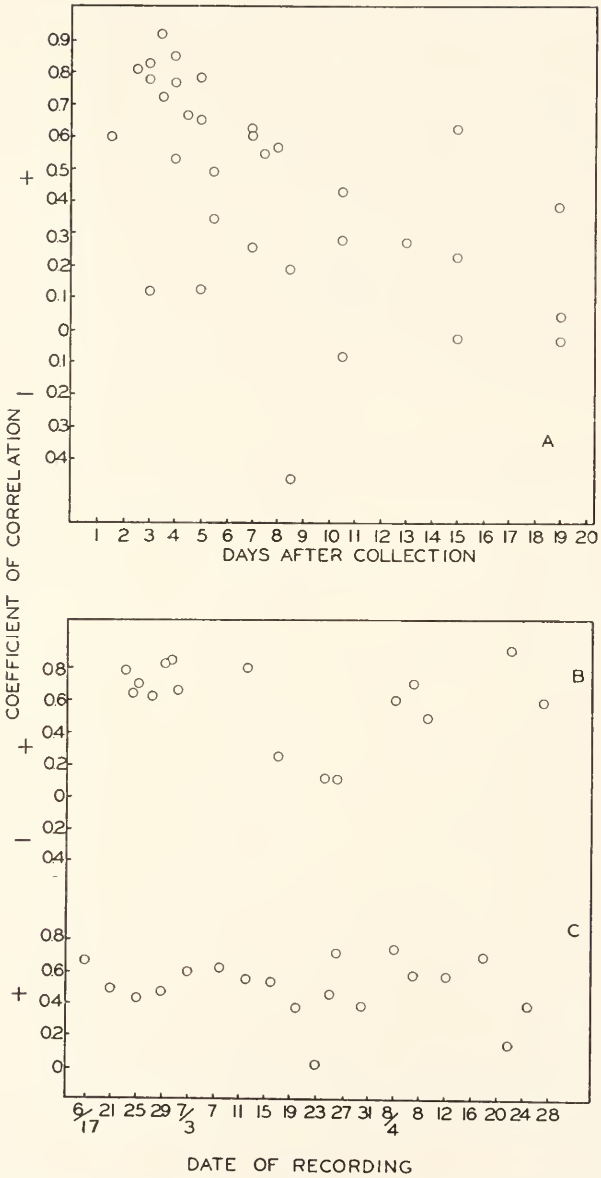

FIGURE 8. Coefficients of correlation of simultaneously recorded values of O_2-consumption for different groups of *Uca pugnax*, (A) as a function of duration of stay in laboratory, (B) as a function of date of collection, (A and B for 1957), and (C) as a function of date of collection (data from 1956).

time difference between the curves compared. The progression of maxima and minima throughout the day seen in Figure 5 for a single semi-lunar period is seen, in Figure 6, to have been repeated throughout the entire summer.

The striking similarity of the form of curves representing comparable times of the semi-lunar period is further illustrated by a comparison of the results obtained in different years. In Figure 7 are plotted the mean curves for every second day of a semi-lunar period. The points were calculated as deviations from daily means. The top curve of each pair represents the mean of all the indicated days from 1956, the lower member of each pair the mean of comparable days from 1957. With respect to form of the curve and the placement of major maxima and minima the curves for the two years are practically indistinguishable. It is thus clear that a characteristic pattern of fluctuations in respiratory rate is exhibited by *Uca pugnax* and that, although the pattern varies in a regular manner with the semi-lunar period, both the basic pattern and its regular variations were almost identical in the summers of 1956 and 1957.

With the establishment of the existence of a regularly repeating pattern of respiration it became of interest to investigate the extent of agreement within groups of animals at any one time. For this purpose correlations of simultaneous hourly values for O_2-consumption of different groups of animals were performed. The coefficients of correlation were calculated for limited periods of one to three days and were calculated separately for data from animals collected at different times. This method of analysis permitted comparison of results in terms of the actual dates of recording, the duration of time in the laboratory, and the dates of collection. The resulting comparisons are presented in Figure 8 and in Table I.

TABLE I

Comparison of simultaneously recorded respiratory values

Collection date	Coefficients of correlation Days after collection						
	1	3	4	5	7	8	15–17
6/20		+0.779	+0.652	+0.700	+0.625	+0.565	
6/26		+0.825	+0.849	+0.666			+0.226 ±0.12
7/10		+0.808			+0.256 ±0.11	−0.464 ±0.11	−0.022 ±0.12
7/21		+0.120 ±0.10		+0.125 ±0.10		+0.185 ±0.10	
8/3	+0.600		+0.726	+0.490 ±0.06		+0.545	+0.618
8/20		+0.915			+0.602		

In Figure 8A the coefficients of correlation for all of the data obtained in the summer of 1957 are plotted as ordinate values with the number of days in the laboratory along the abscissa. There is, in general, a decrease in correlation with time in the laboratory, such that after eight days the values approach a random distribution. It is also obvious that there is a high degree of scattering of values even when the number of days in the laboratory is small.

Figure 8B shows the coefficients of correlation for simultaneous hourly values of O_2-consumption obtained within the first seven days after collection. These values are plotted as a function of the date on which respiration was recorded. In this graph the low values are clearly grouped in the last two weeks of July while strong positive correlations are found during the rest of the summer.

In Figure 8C are plotted similar coefficients of correlation calculated from the data for the summer of 1956. During that summer animals were collected weekly and none were used after being in the laboratory for seven days. Here, too, there is a drop in the value of the coefficients of correlation during the same period for which it was found in 1957. During 1956 there was, in addition, a reduction in correlation in the latter part of August. Unfortunately there are insufficient data for these dates in 1957 to confirm or deny the existence of a similar reduction in that year.

TABLE II

Correlation of hourly values for successive semi-lunar periods

Day of semi-lunar period	Coefficients of correlation	
	1956	1957
A-1	+0.232±0.10	+0.550±0.08
A-2	+0.618±0.06	+0.485±0.08
A-3	+0.568±0.07	+0.667±0.05
A-4	*+0.075±0.14*	+0.530±0.07
A-5	+0.455±0.09	*+0.040±0.10*
A-6	+0.610±0.06	+0.402±0.08
A-7	*+0.150±0.12*	+0.428±0.09
B-1	+0.518±0.08	+0.430±0.09
B-2	+0.390±0.12	+0.502±0.09
B-3	+0.660±0.06	+0.360±0.09
B-4	+0.230±0.11	+0.610±0.06
B-5	—	+0.440±0.08
B-6	*+0.100±0.11*	*+0.020±0.10*
B-7	+0.440±0.09	+0.430±0.08
B-8	+0.440±0.12	+0.740±0.06

In Table I are recorded values for the coefficients of correlation from each collection made during 1957. The coefficients are listed as obtained for various short intervals after collection up to eight days and for the fifteenth and seventeenth days after collection. A comparison of the values for the third day after collection shows that the only group of animals not showing a strong positive correlation, highly significantly different from zero, was the one collected on July 21. It is also of considerable interest that the group collected on July 10, although showing the usual strong positive correlation on the third day, no longer shows a significant degree of correlation by the seventh day and actually shows a significant negative correlation on the eighth day. This is in marked contrast with the collections of June 20 and August 3. In this latter case a strong positive correlation was still found at seventeen days after collection. It appears, then, that considerable variation in both the initial coefficient of correlation and the rate of decrease of the coefficient is found among the various groups of animals. The variations observed are not obviously related either to phase of moon or to treatment related to maintenance in the laboratory. The only factor that seems to give any system to these variations is the date of recording respiration.

Coefficients of correlation were also calculated for the hourly values on one day of a semi-lunar period with the hourly values for each of the comparable days of successive semi-lunar periods throughout the summer. These provide a measure of the similarity between comparable days in successive semi-lunar periods for five such periods for the summer of 1957. These values are presented in Table II from which it is seen that for thirteen of the fifteen days, coefficients ranging from 0.358 to 0.740 were obtained. For the other two days the coefficients of correlation were found to be not significantly different from zero. The two days for which significant positive correlations did not obtain were the day of new or full moon (day A-5) and the eighth day after new or full moon (day B-6). Similar coefficients of correlation were calculated from the data for 1956 and are included in Table II. It will be observed that of the days for which sufficient data were available to permit making the correlations, days B-6, A-4, and A-7 yielded coefficients not significantly different from zero.

Discussion

Two apparently conflicting characteristics of the behavior of *Uca pugnax* emerge from the results reported in this paper. We find that when population samples, ranging in size from 8 to 24 animals, are compared at 15-, 30-, and 45-day intervals the pattern of respiration is being reproduced almost identically every 15 days. When data from somewhat larger numbers of animals are compared from year to year the same precise reproduction of pattern is observed. However, when a group of four animals is compared with two to five other groups of four animals at the same time, as was done by the correlation of simultaneous hourly values, extreme variability is found. It should be emphasized that there are at least three possible situations, all of which would result in a lack of good correlation. One possibility, of course, is that there are in fact only negligible or random fluctuations in the respiratory rate of all animals. This possibility can almost certainly be excluded. In the latter part of July when conspicuously low coefficients of correlation were obtained, the mean daily curves were of normal amplitude and phase relations. An example of this can be seen in Figure 3, curve E, the points of which represent the mean hourly values for July 23.

A second situation that would lead to poor correlation among simultaneous hourly values would occur if one or two machines were recording the normal rhythmic pattern while three or four were producing non-rhythmic fluctuations or no significant fluctuations.

The third possibility leading to lack of correlation is that different samples, while still rhythmic, have become out of phase. Examination of the individual records revealed one two-day period (July 18–19) when two recorders showed typical high-amplitude fluctuations but with one recorder almost precisely in opposite phase to that of the other. This situation is reflected in the large negative correlation (−0.464) recorded in Table I for the collection of July 10. In no other case was a situation of this type obvious. However, it is recognized that if the individuals making up a sample of four were out of phase with each other the record would be indistinguishable from that produced by four non-rhythmic individuals. The multiple peaks evident in Figure 5B, Curves D and E, may indicate such a lack of synchrony among individuals within samples.

Even though there is no way of distinguishing between a loss of rhythm by part of the population and a loss of synchrony, the fact remains that a sufficiently great proportion of the population retains a rhythm in normal phase relations to impart to the mean daily curves a striking regularity. The question of the basis for this regularity represents one of the fundamental problems in biological rhythms.

For the study of this basic problem there are certain distinct advantages to be gained by examining rhythms with periods other than twenty-four hours. If the rhythm being studied is clearly of semi-lunar (or tidal) frequency there can be no question of induction by fluctuations in environmental factors associated with solar day-night. There is similarly little probability that the normal activity in the laboratory where the animals are kept will exhibit tidal-frequency fluctuations. We are faced then with the situation that a predominant part of the population can maintain the rhythm of O_2-consumption with almost absolute precision for as long as 16 days away from the ordinary tidal influences. This occurred while the animals simultaneously exhibited a diurnal rhythm of O_2-consumption. Either one must attribute a remarkable precision to a biological system in a situation not constant but quite different from the normal habitat of the animals, or one must invoke environmental factors not universally accepted as constituting stimuli for the organisms concerned. The first alternative implies, on the part of the organism, a degree of detachment from the environment that is not entirely acceptable to modern biological thought. The second alternative permits an acceptable degree of dependence upon the environment but requires the recognition of hitherto unsuspected stimulating factors.

SUMMARY

1. The form of the mean diurnal rhythm of O_2-consumption of *Uca pugnax* is described and found to be practically identical for the summers of 1955, 1956, and 1957.

2. The mean lunar-day rhythm of O_2-consumption is described for the summers of the same three years. The curve for any one of these years is indistinguishable from that for either of the other two years.

3. The mean lunar-day rhythm consists of two maxima, of equal magnitude, occurring approximately at lunar zenith and at lunar nadir; between the maxima are two minima symmetrical with respect to time of occurrence and magnitude.

4. For the primary lunar rhythm the ratio of maximum to minimum is about 1.4; for the diurnal rhythm the ratio of maximum to minimum is 1.2 in 1955, 1.4 in 1956, and 1.2 in 1957.

5. Because of the amplitude of the lunar component of the rhythm, the data for single days reveal clearly the progression of lunar maxima and minima.

6. Because of the equality in amplitude of fluctuations correlated in time with lunar zenith and with lunar nadir, the overt rhythm is one with a period of 12.4 hours. There is a pattern of fluctuations characteristic of each day in a semi-lunar period.

7. The reproducibility of the daily pattern in successive semi-lunar periods, and in successive years, is demonstrated.

8. In general a strongly positive correlation is found between simultaneous hourly values for different groups of animals during the first seven days after col-

lection. There is a general decrease in the extent of these correlations with time in the laboratory.

9. There is strong evidence for a time-dependent variable affecting the size of the coefficients of correlation for simultaneous hourly values obtained during a wide range of times in the laboratory.

10. Hourly values for single days of a semi-lunar period were correlated with the hourly values for comparable days of successive semi-lunar periods. The coefficients of correlation were positive and significantly different from zero for thirteen of the fifteen days of 1957, the exceptions being the eighth day after new or full moon and the day of new or full moon for which the coefficients were not significantly different from zero. For 1956 the exceptions were the day before new or full moon, and the second and eighth days after; the coefficients for these three days were not significantly different from zero. For the other twelve days the correlations were strongly positive.

11. The relevance of these findings to an understanding of the phenomenon of biological rhythmicity is discussed.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1958

ABSTRACTS OF SEMINAR PAPERS

July 1, 1958

Complete reconstitution from ectoderm in Cordylophora. Edgar Zwilling.

When ectoderm from Cordylophora coenosarc was cut into a number of small fragments and allowed to fuse with an equal amount of fragmented endoderm, reconstitution was very rapid; the tissues of both layers retained their integrity, quickly separated into two layers and formed a hydranth within 36 hours. When pure ectoderm was isolated or left with a small trace of endoderm reconstitution of an individual, complete with two layers, occurred in many cases after 5–7 days. Characteristically such masses formed a small sphere whose wall was composed of small cells and whose center was filled (within ten hours) with the debris from disintegrated cells. All of the large cells (including the endoderm when it was present) were involved in this disintegration. An inner layer then formed slowly and was evident after four days. In order to be certain of elimination of all possible contaminating endoderm cells a number of masses of ectoderm were set up; the spheres were opened after 10 hours (when the central mass had broken down), the central mass was cleaned out and the small-celled walls from several spheres were cut up and allowed to fuse together. All five of the "second generation" reconstitution masses formed an inner layer after 5–7 days and one of them formed a complete hydranth. Without specifying the particular cell involved, this evidence reveals that endoderm may form from ectoderm in Cordylophora.

Neural and mesodermal hierarchies in chick development. Maxwell H. Braverman.

Specific inhibition: The injection of one-day-old chick eggs with extracts of adult organs, as suggested by P. Lenicque, demonstrates, within a few days, the specific inhibitory effect of these extracts on the corresponding embryonic organ system. (Details of extraction will be found elsewhere in this volume.) Neural fractions affect the neural system; mesodermal derivatives their corresponding systems. Eggs receiving injections of the normal saline carrier of the extracts develop an insignificant number of specific defects.

Cumulative inhibition: A pattern of neural inhibition can be seen if the effects of extracts of different parts of the brain are compared. Fore-brain extracts affect only fore-brain formation. Extracts of whole brains inhibit normal formation of the entire brain structure. Spinal cord extracts, in addition to affecting spinal cord, exert an inhibitory influence on the whole brain. Thus there is indicated a tendency for neural structures to inhibit not only homologous tissues but also any tissue forming anteriorly in the neural system.

Mesodermal inhibition: Tests made using extracts of heart, blood vessels, ureter and kidney show that these extracts can inhibit formation of structures more dorsal in the mesodermal hierarchy of Yamada. Diminution of somite number was the most frequent defect; however, some animals injected with either blood vessel or heart extract lacked all or almost all mesodermal structures.

Contradiction: Some structures, such as neural ganglia and the infundibulum, form normally even when surrounding regions are severely inhibited, suggesting that these receive their developmental cue from outside the neural system. Unlike other brain parts, they do not differentiate as hierarchical alternatives in a self-limited system.

A feed-back mechanism of growth control in tadpoles. S. Meryl Rose.

Several workers have demonstrated that the growth of aquatic organisms can be limited by exhaustion of nutrients and by accumulation of relatively specific products in the medium.

Five to 10 *Rana pipiens* tadpoles, each weighing 0.5 gram, growing in one liter of water previously conditioned by snails, fish, plants and a microflora and microfauna add something to the water within 24 hours which greatly reduces the growth rate of assay tadpoles weighing 0.1 to 0.2 gram apiece. This material can be removed by heating to 60° C., by freezing and thawing, by centrifugation at 2000 × g and in other ways as demonstrated by Richards.

Growth of *R. pipiens* tadpoles is increased by culturing them with other animals: *Triturus, Necturus, Lebistes* and *Physa*. *R. pipiens* are inhibited by *R. catesbiana*. Culture water from starved *R. pipiens* is not inhibitory. As the number of tadpoles cultured in 12-liter aquaria is increased from 14 to 53, the average maximum weight decreases from 5.1 grams to 4.2 grams. However, the maximum weight of the largest 14 is the same whether there are 14, 16, 37 or 53. The differences in averages arise because in the more crowded conditions some are greatly inhibited. These, if removed to uncrowded conditions, can resume rapid growth. When four 0.5-gram tadpoles are cultured with four 0.1-gram tadpoles the smaller ones all stop feeding and die in approximately 15 days. The inhibitory effects of culture water from equal weights of large, medium and small tadpoles tested on small tadpoles is proportional to size.

The indication is that growth of tadpoles in limited volumes of water is controlled by a feed-back mechanism involving the production of relatively specific materials capable of being metabolized away by other organisms.

July 8, 1958

The nature of chromatographic amylose and amylopectin fractions. Frederick A. Bettelheim.

Fractionation of amylose on a double column of aluminum oxide of which the upper part is acidic (pH 4.5) and the lower part is basic (pH 7.8) yields three fractions. Two of these can be obtained: I, by eliminating the basic part of the column; II, by eluting the remainder with acetate buffer (pH 5.8). The third fraction cannot be eluted and its properties might be inferred only. Similarly, three fractions can be obtained by chromatographing amylopectin. Viscosity, osmotic pressure measurements, light scattering of the solutions of the fractions were performed, together with enzymatic digestions coupled with biochemical analyses. The experimental data indicate that fraction I of the amylose is a fairly rigid rod-like structure with the possibility of induced positive surface charges which accounts for its passage through acidic adsorbent. Fractions II and III of amylose are polymers of a more flexible nature with increasing random type of coiling. Fraction I of the amylopectin which passes through acidic adsorbent is composed of compact spherical bodies with small percentage of outer branches and large number of branching points. Amylopectins II and III have an increasingly open shape in that order, *i.e.*, the molecular radius of gyration is increasing and so does the percentage of outer branches, while the number of branching points decreases in this order.

In general, the shape of the molecules in this type of chromatography has a greater influence upon the adsorption characteristics than the molecular weight.

Coordination of ciliary motion and muscular contractions in the gills of Crassostrea virginica. Paul S. Galtsoff.

Ciliary motion along the isolated plicae of the gills of bivalves continues for many hours after the severance of tissues from the body. This fact leads to general conclusion that ciliary activity is not under the control of nerve ganglia. Observations made by using oysters with gills exposed by partial removal of shell, but otherwise intact, show that the ciliary motion along the terminal groove of the gill and of frontal cilia frequently stops following spontaneous contraction of the adductor muscle. The cessation of ciliary motion may be general, involving all demibranchs, or may be limited to a small segment of one terminal groove. The cessation may be complete, lasting from a fraction of a minute to almost an hour, or it may continue but at greatly slower rate.

All observations were made on large, adult oysters, partially or completely spawned. All readings were made with low power binocular. Oysters were kept under strong illumination in frequently renewed sea water. Rate of ciliary motion was measured by recording with a stop-watch the time necessary for a small particle of chalky shell substance to pass a known distance along the edge of the demibranch. Records were taken at one-minute intervals for periods lasting from fifteen to fifty minutes.

Electric shock and pricking of the gill surface and mantle produced no effect on ciliary rhythm. Conclusion is reached that in an intact gill there is direct connection between spontaneous contractions of the adductor muscle and the inhibition of the ciliary motion.

July 15, 1958

Factors and genes in Mormoniella. P. W. Whiting and Sarah B. Caspari.

Among several different eye-color loci, one, called *R*, has two factors, *O* and *S*, which mutate to colorless, *oy*, "oyster" and to scarlet, *st*, respectively. Much less frequently these two factors may mutate to intermediate colors. Rarely mutations occur in two further factors, *M* and *N*, giving dark red eyes. All mutant colors are recessive to wild-type brown. The mutant genes are designated by the color with a number or with the initials of the finder. The factors in the gene formulae are arranged arbitrarily in order *O·S·M·N* with symbols indicating the color. Thus gene *oy*-DR is *oy*·+, *st*-DR is +·*st*, mahogany-605 is +·*mh* and dahlia-846 is +·+·*da*. These are unifactorial genes with one-factor difference from wild type. *Oy*-NH *oy·st*, *st*-426, *da·st*, *oy*-848, *oy·mh* and orange-806, +·*st·mh*, are bifactorial and peach-333.5, *pe·st*·+·*mh*, and *da*-838, *rdh*·+·*da·rdh* (*rdh* = reddish) are trifactorial. Compound females are wild type if the mutant factors of each gene have their wild-type alternatives in the allelic gene, as *da*-846/*pe*-333.5 (+·+·*da*·+/*pe·st*·+·*mh*), but they are mutant type if the same factor is mutant in both alleles, as *da*-GF/tinged-277 (*da*·+/*ti·st*). The states or conditions of the different factors form subseries of *alternatives* within the series of *alleles.*

Mutation of factors essential to fertility and viability has occurred in some eye-color genes. Thus, scarlet female *st*-689/*oy*-423 (+·*st·fsa*/*oy·st·fsa*) and wild-type female *da*-835/*st*-689 (+·+·+·*da·la*/+·*st*·+·+·*fsa*) are sterile having an impairment, *fsa*, of factor *A* which is dominant over lethal-a, *la*. However, scarlet female *st*-841/*oy*-423 (+·*st*·+·*fsb*/*oy·st·fsa*·+) is fertile heterozygous for female sterility in different factors, *A* and *B*. The stock with these last two genes is balanced with fertile males, sterile homozygous females. (Work done under U. S. Atomic Energy Commission contract AT(30-1)-1471.)

Tail regeneration in lengthened and shortened earthworms. Sears Crowell.

These experiments were designed to determine whether in the earthworm, *Eisenia foetida*, the number of segments which regenerate following amputation is controlled by the total number of segments remaining (or some other feature of the worm-as-a-whole) or by some local condition at the place of amputation. The plan of the experiment was to cut two earthworms, one at segment 50, the other at segment 70. The longer anterior end of one was sutured to the longer posterior end of the other; and the shorter ends were similarly joined. This gave two worms, one abnormally long by 20 segments, the other short by 20 segments. Ten to 15 segments were later removed from the posterior end and the subsequent regeneration observed. After no further segments were being added the same worm was tested again by removing the regenerate plus a few of the old segments. The operations themselves were performed by Mr. Reisberg and Mr. Buell.

To date we have 19 cases of regeneration by shortened worms and 7 by lengthened worms. In both shortened and lengthened worms the number of segments which regenerated was a few less than the number removed. In 25 of the 26 cases the length of the regenerate conformed to the expectation based on the hypothesis that the local level of the amputation determines the amount of regeneration.

Motion pictures of some changes in cells induced by x-ray treatments of tadpoles and tetrahymenae.[1] CARL CASKEY SPEIDEL.

Modifications of cell division in tadpoles were induced by single whole-body x-ray treatments. In the regenerating tail tip of the tadpole a few days after suitable irradiation (5–10 kr), many cells reached the mitotic stage. Some completed their division with subsequent differentiation of the daughter cells. Others failed to complete their division, the chromatin often remaining in the prophase stage. Degenerative changes followed. In one case a dividing myoblast suddenly ceased its activity shortly after the metaphase stage.

The protozoan ciliate *Tetrahymena corlissi,* a facultative parasite of tadpoles, was very resistant to irradiation. Some individuals survived, single treatments of 400–700 kr. The survivors exhibited conspicuous temporary effects, such as sluggishness, change to a spheroidal shape, and decrease in feeding activity and in rate of reproduction. Rapid recovery ensued. Repeated treatments caused permanent loss of the micronucleus, a structure rich in deoxyribonucleic acid.

Suitable irradiation of tadpole-tetrahymena combinations favored tetrahymenal invasion. While the radio-sick tadpoles became progressively weaker after the treatment, the more radio-resistant tetrahymenae were not noticeably affected. They multiplied and thrived at the expense of the host tadpole tissues.

Radiation-induced strains of amicronucleate tetrahymenae, even after they had received cumulative doses totaling several million roentgens, were still able to invade and parasitize weakened tadpoles. They seemed somewhat less vigorous in their attack on the tadpoles, however, as compared with unirradiated normal micronucleate tetrahymenae.

JULY 22, 1958

The uptake of radiosulphur during the in vitro induction of cartilage.[2] JAMES W. LASH [3] AND HOWARD HOLTZER.

It has been shown previously that the formation of embryonic vertebral cartilage is dependent upon the presence of the embryonic spinal cord or notochord, in both *in vivo* and *in vitro* development. If, in tissue culture, embryonic spinal cord is placed on one side of a millipore filter with somites on the other side, cartilage forms in the somites on the fourth day of culture. If the spinal cord is removed from the filter after 12 hours of culture, the somites will still form cartilage. This demonstrates that the spinal cord factor has passed through the filter and acted upon the somites within the first 12 hours of culture, even though cartilage does not appear for another three days. If chemical differentiation precedes morphological differentiation during chondrogenesis, chemical evidence of chondroitin sulphate might occur any time after induction prior to the appearance of cartilage. In order to detect the appearance of chondroitin sulphate in minute quantities, radioactive sulphur ($Na_2{}^{35}SO_4$) was added to notochord-somite cultures and the cultures were analyzed for the appearance of radioactive chondroitin sulphate at intervals during the culture period of 6 days. The chondroitin sulphate-protein complex was extracted with the method of Einbinder and Schubert and the activity of incorporated ^{35}S was determined. Chondroitin sulphate was found only when cartilage was visible in culture. Within the limits of detection thus employed, the appearance of chondroitin sulphate and of morphological cartilage are concomitant in occurrence.

Mode of action of choline esters. Substrate specificity of their "receptor-protein." MENACHEM WURZEL.

A series of choline esters applied to a variety of responsive tissues (striated and smooth muscle, heart, salivary gland, blood pressure) demonstrated a potency relative to acetylcholine

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[1] This investigation was supported by a research grant (PHS RG-4326 C) from the National Institutes of Health, Public Health Service.

[2] This research was supported in part by a grant to Dr. H. Holtzer from the National Institutes of Health, Public Health Service (B-493-C4).

[3] Fellow of the Lalor Foundation.

(ACh), that followed one of two orders. (1) Butyrylcholine (ButCh) > propionylcholine (PropCh) > ACh > benzoylcholine (BenzCh) > acetyl-b-methylcholine (MeCh). This is also the order of their relative rates of hydrolysis by pseudocholinesterase (pseudoChE) taken at $pS_{opt.}$. (2) ACh > MeCh > PropCh > ButCh > . . . >> BenzCh and this is the order of hydrolysis rates with true cholinesterase (true ChE) at $pS_{opt.}$, except MeCh and PropCh being interchanged. We called the first order of activity "pseudo ChE pattern," the second "true ChE pattern." We never met a different order of potency, although MeCh in a true ChE-patterned uneserinised organ may sometimes be more potent than ACh, but not if the organ was eserinised.

The above patterns could be simply accounted for if the requirement for biological equipotency would be a given constant reaction velocity of an enzymatic reaction $v = k.E.S$ catalysed by pseudo ChE or true ChE. This means that if the affinity of that enzyme for one particular substrate is small, then a higher S substrate concentration is needed to give the same $v = \text{const.}$ necessary to one given effect. A common end-product of this ester hydrolysis is H^+. This way equipotent choline ester concentrations would be those producing a given amount of H^+ per unit of time.

To make legitimate comparison between the enzymatic rates of hydrolysis and biological potencies, we determined enzymatic hydrolysis curves at low substrate concentrations down to 10^{-6} *M*, in the range of biological effects. Suitable reaction mixtures of enzymes plus substrates were made up and samples taken at time intervals measured on a calibrated frog rectus muscle, guinea pig intestine or other sensitive organs. Curves obtained in this way for human serum pseudo ChE, Torpedo true ChE, eel true ChE were presented. It was seen that the order of concentrations giving the same H^+ production rate gave the orders of their biological potency. The interchanged order of PropCh-MeCh in true ChE patterned organs was discussed and this relatively minor divergence attributed to molecular structure differences which act on some factor independent of the enzyme.

July 29, 1958

Ecological isolation and independent speciation of the alternate generations of plants. Albert J. Bernatowicz.

Hutchins (1947) theorized that, since temperature affects both reproduction and survival, at least four combinations of limiting temperatures must be considered in comparing the north-south distribution of species. From the viewpoint of plant biology two complications arise: 1) vegetative reproduction is so common that re-population may be possible wherever survival of individuals is possible, even if critical temperatures for sexual or sporic reproduction do not occur in some parts of the range; 2) it is possible that gametophyte and sporophyte generations of a species may have different temperature tolerances. The first possibility implies that some plant species which are basically of Hutchins' zonal types 2, 3, or 4 will become established only as gametophytes or as sporophytes at one or both ends of their ranges, since vegetative reproduction usually does not produce the alternate phase. Likewise, the second possibility suggests that only one phase of a species may persist in an area if reproduction produces an alternate generation which cannot survive the local temperatures. Similar reasoning for salinity as an isolating mechanism can be applied to those algae of the Baltic and Black Seas which persist in only one of their phases, and photoperiod or other ecological factors may operate in like manner for certain species. An implication of this hypothesis of ecologically isolated generations is that genetic divergence or "speciation" of the separated phases can occur if somatic mutations accumulate and result in distinct clones. This would explain the puzzling situation in the algal genus *Derbesia,* of which there are apparently more "species" of sporophytes than of gametophytes.

A technique for the study of the effects of "host-factor" on the behavior of commensal polychaetes and crustacea. Demorest Davenport.

A new technique is described whereby it is possible to analyze the behavior of the individual commensal polychaete or crustacean when influenced by "host-factor." By this technique one can investigate the specificity of response of commensals and at the same time accrue enough quantitative data to demonstrate the presence or absence of chemotaxes, rheotaxes and kineses.

The development of schooling behavior in the genus Menidia. EVELYN SHAW.

Field and laboratory investigations show that schooling in *Menidia* develops gradually. Schooling begins between two and three weeks after hatching following a period during which a characteristic developmental pattern of approach and orientation can be observed. Young fish, 5–7 mm. in length, approach species mates closely and quickly part without orientation. Fish, 8–10 mm. in length, approach closely, assume a parallel orientation and swim together for a short distance. Fish, 12 mm. in length (about the seventeenth day after hatching), approach, orient parallel to one another and maintain a rather constant orientation while swimming for long distances and long periods of time. In this early schooling, the fish-to-fish distance is, at first, highly uneven, ranging from 5 to 35 mm. The members of the school do not move at uniform speeds and they do not always swim parallel to one another. As the members of the school grow the fish-to-fish distance becomes less variable, the swimming speed is more uniform and orientation is more precisely parallel. An explanation for certain aspects of the gradual development of this uniform and precise behavior may be that some experience is required to perfect orientation within the school. In order to evaluate the importance of experience, attempts were made to rear fish in physical and visual isolation. Out of 400 fish, only four grew to 12 mm. in length. When these four were presented to a school of fish of the same size they joined the group immediately. However, initially, they seemed unable to maintain their position in the school; they occasionally swam away from the school and they often bumped into species mates. At the end of four hours, however, fish reared in isolation could not be distinguished from those reared in groups. The results with these four fish agree with the proposition that the precision of orientation within the school is, in part, a learned phenomenon.

AUGUST 5, 1958

Effects of sperm extract and other agents on the egg membranes in relation to sperm entry in Hydroides hexagonus.[1] ARTHUR L. COLWIN AND LAURA HUNTER COLWIN.

Electron microscope studies of thin sections show that the outer covering of the egg, sometimes described as a thick, refractive "vitelline membrane," is in fact composed of at least three distinct entities: (1) a thin *outer border layer* which is complex in structure, (2) a thick *middle layer,* constituting the major portion of the membrane, and (3) a thin *inner border layer* whose underlying structure is somewhat similar to, but denser than that of the middle layer. The appearance of both these layers differs greatly from that of the outer border layer.

Observations and photomicrographs of living material now clearly confirm that a hole or space remains in the vitelline membrane after the sperm head has completed its passage into the egg proper. Following previous electron microscope studies it was suggested (Colwin and Colwin, 1957a) that the spermatozoon produced a lytic agent which could dissolve the membrane, thus forming these holes. It was then found (Colwin and Colwin, 1957b) that extracts of frozen-thawed sperm could rapidly dissolve material *beneath* the outer border layer. Electron micrographs of thin sections of eggs exposed to these extracts now show the extent of the membrane lytic action: only the middle layer is dissolved by the extract; the inner as well as the outer border layer appears to persist. The microvilli of the egg are not affected and are left to project freely following dissolution of the middle layer material which normally surrounds them.

Electron micrographs show no apparent modification of the components of the egg membrane following fertilization and the membranes of fertilized eggs were affected in the same way as those of unfertilized eggs when exposed to the sperm extract.

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[1] Supported by a Grant (RG-4948) from the National Institutes of Health, U. S. Public Health Service.

Fertilization and agglutination inhibitors from Arbacia.[1] CHARLES B. METZ.

In keeping with observations of some others (*e.g.,* E. B. Harvey, 1956) it is found that gametes obtained from *Arbacia* by KCl-injection sometimes give reduced fertilizability. This appears to be related to release of a yellow-green dermal secretion which inhibits fertilization (Oshima, 1921; Pequegnat, 1948). Further study reveals that the dermal secretion has other effects as well. These include an enhancement of sperm motility, a two-fold increase in sperm respiration and inhibition of fertilizin agglutination of sperm.

This last effect suggests that further study of the dermal secretion might provide information concerning a role of fertilizin in fertilization. Experiments show that the dermal secretion destroys the agglutinating action of fertilizin. Thus the inhibiting agent is heat-labile whereas fertilizin is heat-stable. Heated fertilizin-inhibitor mixtures neither agglutinate sperm nor inhibit agglutination. Evidently the inhibitor inactivates fertilizin and is subsequently itself destroyed by the heat treatment. Furthermore, the inhibitor does not inactivate by merely converting fertilizin to the univalent form. It actually inactivates the combining sites of the agglutinin. Thus, sperm washed from inhibitor alone or from inhibitor-fertilizin mixtures agglutinates on addition of fertilizin, whereas sperm washed from fertilizin alone fails to agglutinate. Accordingly, fertilizin fails to block the sperm surface in the presence of the inhibitor. This is explained by assuming that the inhibitor inactivates the combining sites of fertilizin.

The question whether inactivation of fertilizin is related to the fertilization-inhibiting action of the dermal secretion remains to be answered. Moreover, the question is complicated by the fact that immunological analysis reveals three antigens in the dermal secretion, and that the dermal secretion appears to contain a second, heat-stable agent which inhibits agglutination of eggs by antifertilizin from sperm.

Behaviour of metachromatic granules during cleavage in Spisula. LIONEL I. REBHUN.

Eggs stained vitally in dilute solutions of such dyes as toluidine blue, azure A, azure B, and methylene blue in sea water possess small (about ½-micron) granules which, in the case of the first three dyes mentioned, are metachromatic. These granules appear in the unfertilized egg. After centrifugation for 10 minutes at 10,000 g against a sucrose barrier, the metachromatic granules are seen in the mitochondrial layer. If stained eggs are fertilized the particles migrate into the asters at cleavage and after a given cleavage are localized on the peripheral poles of the individual blastomere nuclei. Just prior to the succeeding cleavage the mass of particles divides into two smaller masses, each subsequently outlining an aster of the forming spindle. This behaviour continues until at least the fifth cleavage, beyond which we have not attempted to trace it.

Neither the material stainable with zinc-free janus green B nor that revealed by the Nadi reaction shows the same specific localization change as the metachromatic particles. This presumptive evidence that the particles are not mitochondria is supported by electron micrographs which show that the mitochondria are scattered at random in the egg at cleavage, being excluded only from the spindle.

AUGUST 12, 1958

The A band of muscle from Limulus polyphemus. G. W. DE VILLAFRANCA, T. S. SCHEINBLUM AND D. E. PHILPOTT.

Strips of rest-length muscle from the cephalothorax of *Limulus* were tied to splints and placed in ice-cold 50% glycerol. After deep-freeze storage for about 10 days they were cut from the splints, blended 1½ minutes and washed seven times in 0.04 *M* KCl-0.0067 *M* phosphate buffer (pH 7.4) solution. In five experiments an average of 29.3% of the original protein was left after washing.

The washed fibrils retained their characteristic morphology as judged by phase contrast and electron microscopy. One could observe A, I and Z bands, but no M lines or H zones which are also absent in fresh or fixed material. Filaments about 150 Å or larger and filaments, or rings, of material about 60 Å wide were observed in cross-section. Occasionally filaments were seen running through the Z bands.

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[1] Aided by a grant from the National Science Foundation.

When the washed fibrils were extracted with solutions of high salt, (0.48 *M* KCl, 0.01 *M* pyrophosphate, 0.1 *M* phosphate buffer pH 6.5, and 0.001 *M* $MgCl_2$; or, 0.3 *M* KCl, 0.15 *M* phosphate buffer pH 6.5 and 0.002 *M* ATP) the A band disappeared leaving a ghost fibril with Z band, Z band filaments and 60 Å filaments extending throughout the sarcomere, often in disarray. This is similar to the effect of these solutions on rabbit psoas muscle. There was, however, a striking difference between rabbit and horseshoe crab muscle. Under these conditions actomyosin, rather than myosin, was always the chief protein extracted from the crab. In 10 experiments an average of 38.2% of the washed fibril protein was removed (11.2% of the whole fiber). The extract had an ATP sensitivity (viscosity drop with ATP) of from 65.1 to 165.0. About 70% of the protein precipitated upon dilution with 10 volumes of water and exhibited both the super-precipitation and ATPase reaction of other actomyosins.

Sperm cell models and the question of ATP-induced rhythmic motility. DAVID W. BISHOP.

Several types of KCl-glycerine-extracted cell systems which respond to ATP by rhythmic motility have previously been demonstrated. These systems include grasshopper sperm and Vorticella stalk myonemes (Hoffmann-Berling), toad pharyngeal cilia (Alexandrov and Arrouet) and spermatozoa of several species of mammals (Bishop). Considerable evidence now indicates that rhythmic flagellation is due to contraction-relaxation cycles inherent in the contractile protein present in these cells. Two fundamental problems are posed by the behavior of these ATP-reactivated cells: (1) How do contraction-relaxation cycles continue, in contrast to the muscle fibril model which contracts only once upon the addition of ATP? (2) How can a coordinated wave-like movement occur in cells whose permeability, ionic balance, and metabolic integrity have been destroyed?

A partial answer to the second question has been found in a comparison of the motility of normal cells with that of sperm models. The motility of fresh bull sperm consists of two components: a two-dimensional vibration initiated proximally and propagated along the flagellum, and a superimposed spin along the longitudinal axis due to helical flagellation of the distal region of the tail. Sperm models, on the other hand, display only the two-dimensional bending waves; neither wave propagation nor the three-dimensional spiraling occurs. The lack of these two elements of motion suffices to explain the failure of cell models to undergo forward progression, despite very vigorous movements. Attempts to restore the missing components of flagellation by adjustment of the ionic balance in the models have not proved successful. It is recognized that the disrupted processes discussed here are perhaps more involved with intracellular co-ordination than with the chemistry of wave mechanics, but such a study permits a recognition of those aspects of motility which are and are not concerned with the movement of reactivated, extracted sperm models.

ATP—an energy source for sperm motility.[1] LEONARD NELSON.

Morphological features of motile systems as well as ATP-ase activity and contractility must be taken into account in theories concerning mechanisms of motility. Perhaps more than a superficial similarity exists between the submicroscopic organization of sperm of warm-blooded animals and the elegant Huxley-Hansen interdigitating creep arrangement of muscle. Following appropriate fixation and staining of sperm, connections appear to link the nine outer longitudinal fibers with the corresponding nine inner fibers. Reversible changes in alignment of these structures during generation of the undulatory wave would presumably be associated with release of energy. The average power developed in the propulsion of bull sperm through a viscous medium, calculated as a first approximation using Carlson's formula, is about 3.15×10^{-8} erg/second. To yield this amount of energy, an average sperm must split 1×10^{-19} *M* ATP/second. At a Q_P of 150, the spermatozoon can avail itself of a "safety factor" of two orders of magnitude. Succinic dehydrogenase activity can regenerate at least twenty times

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[1] Supported by grants from the Population Council, Inc., New York.

the amount of ATP utilized. Electron microscope cytochemical studies suggest an organization which can meet the requirements imposed on an undulatory filament propelling itself by three-dimensional waves. The micrographs reveal an association of both ATP-ase and succinic dehydrogenase within the nine outer fibers. To determine whether these fibers contain substances related to known contractile proteins, frozen-dried sperm were incubated in solutions containing myosin antibodies. Electron micrographs consistently show increased density of the nine outer fibers. Perhaps the fibers bind the antibodies weakly, since results were obtained only when unorthodox procedures were employed. If propulsion of mammalian sperm depends on sequential "contraction" of the outer fibers, this may be achieved by a lateral "creeping" along the inner fibers. The initiation and regulation of the propagated waves remains to be investigated.

Polarization optical studies on amebae. ROBERT D. ALLEN.

It has been our hope that analysis of the cytoplasm of ameboid cells with polarized light might yield useful information on the molecular mechanisms involved in ameboid movement. The sign and magnitude of birefringence and rotation can reveal not only molecular orientation, but sometimes designate the molecules involved and provide information on their conformation. Earlier attempts have been hindered by (1) lack of sufficiently sensitive methods for measuring small retardations and angles of rotation, and (2) the presence of crystalline cytoplasmic inclusions which scatter and depolarize light. Recent advances in the design of polarizing microscopes (Inoué) have made it feasible to re-investigate ameboid cells by a new method employing objective recording of small intensity differences in the presence of scattered light. In this method, suggested by Dr. Inoué, the plane of polarization of the incident beam has been wobbled by a rotating, tilted, strain-free, optically-flat cover-glass. When the polars are crossed, the photomultiplier records for each revolution of the tilted cover-glass four equal intensity modulations, the peaks of which correspond to the positions in which the axis of tilt forms an angle of ± 45° with the plane of polarization. Various model experiments have shown that either small retardations or small angles of rotation modify the recorded intensity pattern. In the present experiments to measure birefringence, retardation could be separated from rotation by the orientation of the specimen, since a birefringent object shows opposite intensity patterns at plus and minus 45° settings. Using a 20° tilt, retardations of 0.5 Å could be detected with monochromatic light.

A spot of wobbling linearly-polarized light was directed at the anterior portion of a somewhat compressed monopodial ameba (*Chaos chaos*). As the specimen advanced, the light beam scanned its long axis, recording retardations in the front, middle and rear portions as well as a control for the strain birefringence of the cover-glasses (maximum 2 Å). The endoplasmic stream was positively birefringent (10^{-6} to 10^{-5}), with the highest values recorded in the middle and lowest near the tail. Although birefringence seemed to depend on the rate of streaming, it would be premature to call this flow birefringence, as it has not yet been established whether streaming or some other factor associated with movement brought about the inferred alignment of protein molecules.

The isolation and analysis of cilia. FRANK M. CHILD.

In order to speculate seriously about the mechanism of the movement of cilia and flagella it is necessary to know something about the molecular organization and chemical composition of these cellular organelles. Such information is obtainable by isolation and analysis of the structures themselves.

Cilia have been isolated from the protozoan ciliate *Tetrahymena pyriformis* in quantities sufficient for analysis. The cilia are isolated from the living cell directly into 20 per cent glycerol. Electron micrographs show that isolated cilia are composed of the fibrillar axonemes. Isolated cilia are insoluble in water, KCl solutions, Versene, 6 *M* urea, and within the pH range 3 to 11. The substance of cilia that have been dissolved at a pH greater than 11 remains in solution down to pH 5.4. Dissolved ciliary substance shows but one electrophoretic and ultracentrifugal component in phosphate buffer, pH 7.4. Ciliary substance is largely protein, but has a U.V. absorption maximum at 260 mμ in acid which is attributed to the presence of adenine and uracil nucleotides since the material contains phosphate, pentose, adenine and uracil.

RNA is not present. Calculations based on the pentose determination indicate that the amount of the nucleotide is about 2.5% that of the protein. The nucleotide is not separable from the protein ingredient by precipitation at pH 5.4, nor by prolonged dialysis. However, the nucleotide does not sediment proportionally with the protein when ultracentrifuged. Isolated cilia possess enzymatic activity which will split phosphate from adenosine triphosphate, adenosine diphosphate, and adenosine-5′-monophosphate.

AUGUST 19, 1958

A physical study of the ground substance of the Spisula egg. L. V. HEILBRUNN AND W. L. WILSON.

As is well known, the most widely-used method of measuring protoplasmic viscosity is the centrifuge method. In this method, cells are centrifuged and the speed of movement of protoplasmic granules is determined. According to Stokes' law, this speed gives a measure of the viscosity. But in determining relative values for the viscosity of the hyaline protoplasm, it is essential that the size of the granules, their specific gravity and their number or concentration remain constant.

It has often been reported that in immature invertebrate eggs, the protoplasmic viscosity is very high and decreases markedly following the breakdown of the germinal vesicle. In the eggs of the clam, *Spisula solidissima,* after breakdown of the germinal vesicle, granules move through the protoplasm much more readily. However, when following centrifugation, the speed of return of the granules is measured, and Einstein's formula for Brownian movement is applied, it can be shown that the viscosity of the hyaline protoplasm or ground substance is essentially the same before and after germinal vesicle breakdown. Thus a series of 14 measurements gave a value of 5.0 centipoises for the viscosity of the ground substance before germinal vesicle breakdown, whereas 13 measurements gave a value of 4.7 centipoises after breakdown. When the large germinal vesicle breaks down, the concentration of granular material in the cytoplasm becomes much less—also new smaller granules appear in the cytoplasm. Both these changes are primarily responsible for the fact that the non-granular (hyaline) zone of centrifuged eggs appears much more rapidly when eggs are centrifuged after germinal vesicle breakdown.

According to the equations of Einstein and of Simha for the viscosity of suspensions, the viscosity of the entire protoplasm is decidedly greater in immature eggs, but this does not appear to be true for the hyaline protoplasm or ground substance.

Physical properties of lobster nerve axoplasm. CARL FELDHERR.

The physical properties of giant lobster axons were studied with the aid of microinjection methods. When small drops of paraffin oil (4–40 μ) were injected into the axons, the drops did not move under the influence of gravity as long as the axons were capable of transmitting an impulse. However, when as a result of injury or aging, the axons had lost their ability to conduct, oil drops did move readily through the axoplasm. This indicates that in the normal axon there are structural elements which break down after injury. The injected oil drops were always spherical, whereas according to Chambers and Kao oil drops injected into squid axons assume an ovoid shape. Apparently squid axoplasm is more rigid than similar axoplasm in the lobster. If large amounts of oil are injected into lobster axons, the oil appears to fill the entire width of the axon, indicating that if a cortex is present, it could be no more than a few microns thick. Electrical stimulation of the axon produced no change in the shape of injected oil drops. By injecting a relatively large amount of oil, changes in the shape of the spike could be produced. These changes were usually found to be reversible upon removal of the oil.

Studies were also made with phase contrast microscopy, and these gave results similar to those previously obtained by Tobias and Bryant. Vigorous Brownian movement could always be observed, and there was a vibratile movement of filamentous structures scattered through the axoplasm. In some instances fibrils could be observed in the center of the axon; these were not as extensive as those described by de Rényi, and their presence was not necessary for conduction, for in those cases in which no fibrils could be observed, the axons were able to transmit impulses.

The nuclear envelope as a possible agent in specific synthetic events in the cytoplasm of sand dollar eggs. ROBERT W. MERRIAM.

Late in the process of vitellogenesis of *Dendraster eccentricus* oocytes, double membranes scattered singly in the cytoplasm can often be seen in electron micrographs in intimate, parallel association with the envelope of the germinal vesicle. The membranes resemble the nuclear envelope and often have 150–200 Å particles adhering to the surface. Concentrically arranged double membranes of "yolk nuclei" are morphologically similar, including 150–200 Å adherent particles. Such membranes show no sign of regularly arranged annuli.

After the maturation divisions, the mature nucleus becomes massively associated with closely applied and parallel arrays of the annulate lamellae named by Swift. At the same time intranuclear vesicles can be seen in structural continuity with double membranes parallel to, but inside, the nuclear envelope. The "intranuclear membranes" are always closely applied to the nuclear envelope and are morphologically identical to it except that no annuli are present.

Such intimate association with the nuclear envelope, coupled with their morphological similarity to it, are taken as evidence suggesting that single granulate membranes, perhaps "yolk nucleus" membranes, and annulate lamellae are somehow formed around the nuclear envelope.

Annulate lamellae are shown to contain regularly arranged annuli consisting of electron-dense rims in which relatively less dense spheres or vesicles are embedded. Masses of 150–200 Å, basophilic granules are found closely associated with some of them in the cytoplasm. These are the "heavy bodies" of Afzelius. "Heavy bodies" are never observed to be structurally associated with the nuclear envelope. Therefore, it is suggested that 150–200 Å particles are formed by annulate lamellae in the cytoplasm.

If the 150–200 Å particles of the "heavy body" are homologous to the particles of Palade, the annulate lamellae may form nucleoprotein structures which contribute to the cytoplasmic synthesis of proteins. This suggests that synthetic specificity, perhaps originally derived from the nucleus, may reside in nuclear envelope structure.

ELECTROBIOLOGY SEMINARS

JULY 10, 1958

Graded electrical responses HARRY GRUNDFEST.

Electrically excitable electrogenic cells that normally produce regenerative (all-or-none) spikes become gradedly responsive in early relative refractoriness, during sustained depolarization, or after treatment with synapse inactivator drugs. In the grasshopper, *Romalea microptera,* electrically excitable muscle fibers have only graded responsiveness. This finding confirms an earlier suggestion; the combination of different grades of electrically inexcitable postsynaptic potentials with graded responsiveness of electrically excitable fibers of insect and crustacean muscles underlies the variety of their electrical and mechanical responses to different axons of their innervation. Normal occurrence of graded responsiveness indicates that current theory, devised to account for production of spikes, is inadequate as a general account of electrically excitable electrogenesis. A preliminary hypothesis views electrically excitable electrogenic membrane as a composite of unit areas, each endowed with a complement of "electrogenic elements" which may be pores, carriers, or other mechanisms for changing ionic conductances when appropriately triggered by some (depolarizing) electrical stimulus. The elements of a unit area are considered to have thresholds requiring different intensities of triggering stimulation. Distribution of this population in a narrow range with respect to their thresholds (equivalent to a high amplification factor in a bistable electronic analogue), leads to regenerative action within the population, and to all-or-none responses. Widespread distribution of the population on the threshold axis would tend to eliminate regenerative action by electrotonic losses, and could result in graded responses. This also occurs in the electronic analogue with reduced amplification. Wide-band distribution of electrogenic elements is considered to be the normal case in the invertebrate muscle fibers and to be produced physiologically or pharmacologically in converting regeneratively acting to gradedly responding tissues. The hypothesis clarifies experimentally observed distinctions, blurred by the current ionic theory, between the capacity for electrogenic activity and the initiation of this activity by appropriate stimuli. It has also predicted an experimentally verified relation between area of stimulated membrane and the character of responses in gradedly responding eel electroplaques. When the whole electrically excitable surface is stimulated, the response appears to be a spike.

July 17, 1958

The membrane potentials during rest and activity of the electroplate of Raia clavata. Rosamond M. Eccles and L. G. Brock.

The electrical responses of individual electroplates were investigated with intracellular micropipettes in organs isolated from *Raia clavata.* At high potassium concentrations there was a linear relationship between the logarithm of the concentration and the resting potential, with a gradient of 58 mV for a ten-fold increase in potassium concentration. The resting potentials which were in the range of 60–70 mV were unaffected by changes in extracellular magnesium, calcium and chloride ions. The response to stimulation in many ways resembled the amphibian endplate potentials. At its peak the voltage was usually close to the zero potential line (± 10 mV). The response reached its maximum in 3–5 msec. and declined to the resting level in about 25 msec. In low concentrations of sodium chloride there was a marked increase in the duration of the response. Alterations in extracellular chloride levels seemed to indicate that movement of chloride ions was not important in the recovery process. The blockage by curare and the prolongation by prostigmine lend support to the belief that transmission at the nerve-electroplate junction is cholinergic.

Electric organ electrogenesis in Malapterurus electricus. M. V. L. Bennett, R. D. Keynes and H. Grundfest.

The African catfish presents apparently aberrant features among electric fishes. Discharging electroplax become negative at their rostral, uninnervated faces. They were considered to be derived from gland, to be electrically inexcitable, and to hyperpolarize during activity. This explanation appears unlikely. The synaptic junction is at the tip of a long caudal stalk. The postsynaptic potential, electrically inexcitable and non-propagating, therefore cannot involve the major part of the electroplaque, yet, each cell apparently generates a high emf, which indicates this involvement. Microelectrode recordings from single electroplax provide a more satisfactory explanation, furnishing also new data for the general theory of bioelectrogenesis. The electroplax are electrically excitable, the impulse synaptically evoked in the stalk propagating into and activating the cell body. Responses of the latter to direct stimulation do not differ from neurally evoked activity. Electrogenesis of the caudal as well as the rostral face contributes to an overshooting spike, but the caudal activity is smaller, higher threshold and briefer (*ca.* 0.3 msec.) than that of the rostral face, which lasts about 2 msec. The intracellularly recorded spike consequently has a brief initial peak, when both generators are active, followed by a smaller, longer-lasting portion when only the rostral face continues activity. However, the responses recorded outside the two faces, representing the difference between the two electrogenic activities, have a reduced potential while both faces are responding. The rostral face is negative, the caudal positive during the entire discharge. The neurally evoked activity of a single electroplaque thus must comprise at least four components: a p.s.p. at the stalk-tip, giving rise to an electrically excitable response propagating in the stalk, the latter in turn producing the two activities in the rostral and caudal faces of the electroplaque. This electrogenic diversity, which accounts for the peculiarities of *Malapterurus* electric organ, is greater than has hitherto been found elsewhere.

August 7, 1958

Aspects of synaptic transmission in the squid stellate ganglion. S. H. Bryant.

The synaptic excitation of the stellar giant axons and recent studies supporting a chemical transmission process at the distal (giant) synapses were reviewed. Evidence for, and the properties of, the proximal (accessory) excitatory synapses were given.

New data were presented showing the effects of osmotic pressure, ions, fatigue and temperature on synaptic delay in perfused excised ganglia. Different methods of evaluating synaptic delay were compared and found to agree closely if the same reference points were used. Values from peak of preaxon artefact to the beginning of the psp are given here. Only temperature variation produced marked changes in the delay at the distal synapse. In the range from 35 to 20° C. the delay was relatively constant, averaging 0.55 msec. Below 20° C. there was an accelerated increase in delay up to 1 to 3 msec. near 2° C. Further temperature decreases resulted in block. Similar temperature effects were noted in proximal synapse latency. The constancy of the delay in the higher temperature range is consistent with the notion of transmitter diffusion time. The reason for the large increases at lower temperatures is not clear. High gain intracellular post axon recordings of the presynaptic spike "leak through" in the latter studies most often show the distal to be diphasic and the proximal triphasic, resembling first and second derivatives of the pre spike, respectively. The amplitude of these potentials decreases rapidly with distance from the synaptic area.

Further attempts to detect a quantal nature of the distal p.s.p. were unsuccessful. Fatigue, cold, high os.p., high calcium-low magnesium medium and smaller distal synapses were used to increase this effect if present.

Compounds not used previously were tested for action on the distal synapse. FMN, thiamine, asparagine, hydroxylamine (all at 10^{-3} gm./ml.) and ouabain (10^{-5}) were without effect in 30 to 60 minutes. Picrotoxin (10^{-3}) and choline chloride (2×10^{-2}) caused a reversible non-depolarizing block within 20 minutes. Guanidine (4×10^{-4}) reversibly produced repetitive presynaptic discharges to a single shock, prolonged p.s.p.'s and eventual synaptic blockade.

August 14, 1958

Electroplaque activity in marine electric fishes. M. V. L. Bennett, M. Wurzel, E. Amatniek and H. Grundfest.

Microelectrode recordings prove that electroplax of *Astroscopus guttatus, Narcine brasiliensis,* and *Torpedo occidentalis* respond only to stimulation of their nerves and to chemical agents. Denervated electroplax in *A.*, though activated by drugs, do not respond to electrical stimuli. A constellation of properties (most extensively studied in *A.* and *T.*) associated with electrically inexcitable electrogenesis is found: *i*) stimuli to the innervated surface of an electroplaque (dorsal in *A.*; ventral in *N.* and *T.*) evoke responses after an irreducible latency, about 1 msec. Responses develop only at the innervated surface, are depolarizations from a resting potential of 50–80 mv, last 5–8 msec. *ii*) Responses may be graded into several discrete steps. *iii*) At maximum they approach an "equilibrium potential" which approximates zero membrane potential. *iv*) The amplitude is graded without change in latency by changing membrane potential, increasing with hyperpolarization. *v*) Responses are inverted on reversing membrane potential. *vi*) Different axons apparently innervate discrete regions of the large surface (about 1 cm.^{2} in *A.* and *T.*). Responses produced in one region do not propagate actively into other zones of innervation. Electrotonic spread decays to half in about 0.5 mm. (*T.*). *vii*) Potentials evoked by the same or by different axons sum non-algebraically, addition becoming limited as depolarization approaches the equilibrium potential. *viii*) Homosynaptic facilitation occurs, but not heterosynaptic. *ix*) Without anticholinesterases, acetylcholine depolarizes only in high concentrations (10^{-2} *M* in *T.*). When protected, its threshold effectiveness is at 10^{-4} to 10^{-5} *M*. Carbamylcholine is equally effective without protection. *x*) Anticholinesterases also depolarize and prolong responses, while curare blocks activity without depolarizing. *xi*) Quaternary drugs act only when applied to the innervated side, electroplax below the exposed layer remaining unaffected. However, eserine acts from either side and also affects underlying units. Since *Raia* electroplax are also electrically inexcitable (Eccles and Brock, above), the electrogenic membranes of electroplax in all marine electric fish, teleost and elasmobranch, respond only with post synaptic potentials. However, electrically excitable, spike generating membrane also occurs in electroplax of all fresh-water electric fishes studied thus far, 5 species of Gymnotids and *Malapterurus*.

August 21, 1958

Electrophysiology and pharmacology of lobster muscle fibers. H. Grundfest, J. P. Reuben and W. H. Rickles, Jr.

Studies with intracellular microelectrodes analyzed drug- and ion-induced alterations of lobster muscle fiber membrane. The component that gives rise to inhibitory postsynaptic potentials (ipsp's) is activated by GABA (γ-aminobutyric acid; threshold concentration about 10^{-11} M), β-alanine, β-hydroxy-GABA and γ-aminocrotonic acid. Slight hyperpolarization, four-fold or larger increase of membrane conductance, and appropriate electrochemical modifications of epsp's and ipsp's evoked by stimulating the excitatory and inhibitory axons denote this action on the synaptic membrane. Picrotoxin selectively inactivates the hyperpolarizing membrane without changing conductance. GABA antagonizes its effects. Thus, some degree of inversion is evidenced between pharmacological properties of crustacean hyperpolarizing synapses and depolarizing synapses of cat cortical dendrites, since GABA activates the former, but blocks the latter, while picrotoxin inactivates the former and excites the latter. On the other hand, carnitine activates epsp's of the muscle fibers, depolarizing as well as increasing membrane conductance. GABA antagonizes the depolarization, but increases conductance further. Therefore, the depolarizing and hyperpolarizing synapses are pharmacologically distinct and act independently. Serotonin and histamine depolarize, but in high concentration. Even in 1% solutions, other drugs that activate or inactivate many vertebrate synapses are without effect (*e.g.,* acetylcholine, prostigmine, curare, hexa- and decamethonium, strychnine, and the C_6 and C_8 ω-amino acids). Substituting Rb^+ for external K^+ increases membrane resistance, but augments ipsp's even more. Applying Ba^{++} (with depletion of Na^+) increases membrane resistance about ten-fold. This does not prevent subsequent actions of synaptic drugs and presumably does not affect synaptic membrane. The IR drop resulting from the emf of electrogenic units that are active during the normal graded electrically excitable response should be increased when the resistance is increased by Ba^{++}. The higher potential should activate more units of the electrogenic population (Grundfest, above), and should give rise to regenerative involvement of the whole population. This process satisfactorily accounts for the conversion, in the presence of Ba^{++}, of the graded electrically excitable responses of invertebrate muscle fibers into spikes.

GENERAL SCIENTIFIC MEETINGS

August 25–28, 1958

Abstracts in this section (including those of Lalor Fellowship Reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

Retinal development and phototactic responses in developing Ameiurus embryos. P. B. Armstrong.

The first indication of the rods and cones in developing Ameiurus is seen at the time of hatching when small protoplasmic buds form in the cells of the external nuclear layer in the central part of the retina. The cells of the pigment epithelium are heavily charged with melanin pigment and fill the area between the external nuclear layer and the chorioid.

Twenty-four hours later the receptor layer is broader and the protoplasmic buds have extended and enlarged enough so that it is possible to differentiate the rods and cones from each other. These receptor elements are still broadly attached to their respective cell bodies. The pigment of the pigment epithelium is only sparsely distributed between the receptor elements. It becomes very dense as one proceeds toward the chorioid.

There is a gradual development of the myoids of the rods and cones. Photomechanical changes with varying light intensities are well developed shortly before the complete absorption of the yolk. This includes not only shortening and lengthening of the myoids of the rods and cones but movement of the pigment in the pigment cell layer.

Accompanying the above developmental changes are changes in the motor responses of the developing embryos to light. Further investigations will be necessary to correlate the developmental picture and the behavioral responses.

Electron microscopic investigation of the structure of hyaluronic acid gels and hyaluronic acid-protein complexes. Frederick A. Bettelheim and Delbert Philpott.

Hyaluronic acid (H.A.) was isolated from human umbilical cords. It was purified progressively from proteins, neutral polysaccharides and sulfated polyuronides. Its molecular weight (ultracentrifuge) was 77,000. It was homogeneous, free from contaminations.

Electron micrographs showed that the pure H.A. formed by precipitation with ethanol is a three-dimensional network of microfibrils which organize themselves into platelets. These platelets combine to form needle-like superstructure. X-ray diffraction proved that the H.A. gel is highly crystalline.

Electron microscopic studies of H.A. gel in its complexed form with intercellular proteins were performed on samples taken in the different stages of the purification of H.A. The data reveal that the association with collagen fibers is a loose one and the real complexing of H.A. occurs with non-fibrous proteins. The H.A. platelets are sandwiched between layers of proteins.

H.A.-protein complex model system was constructed by using pure H.A. and blood albumin. Complexes were made at different pH's and at different concentrations. At high pH's no complexing occurs. At physiological pH the complex has a fibrous structure which contracts to a spherical shape when one goes through the isoelectric point (4.3) (which is, however, not the isoelectric point of blood protein). The size of these spheres is directly proportional to the blood albumin/H.A. ratio.

Potassium contracture in a variety of conditions. D. M. Conway and A. I. Csapo.

The retractor penis muscle of the turtle (*Chrysemys picta*), suddenly immersed in excess K Ringer of 20–100 mM/l., goes into a reversible contracture. Activation here is generally explained by the depolarising effect of K, and the [K] of 25 mM/l. is looked upon as the threshold concentration for contracture.

If the [K] of 20–50 mM is gradually rather than suddenly raised, however, no contracture develops.

The turtle muscle can be depolarised suddenly by excess $K = 20$ mM or gradually by excess $K > 20$ mM without any sign of activity, and then activated later by different means, which involve no change or an increase rather than a decrease in membrane potential. If the K is not excessive the muscle can be stored in this "primed" condition. There is no true threshold for K-contracture, and it will develop after treatment of the resting muscle with gradually increasing $K < 50$ mM, if the subsequent jump in [K] is large enough.

Sudden treatment with excess K results in more contracture tension in a propagating than in a non-propagating muscle. Lowering the temperature decreases the "threshold" and increases the magnitude of contracture. Short treatment with Ca-free Ringer increases the slope of rise and the magnitude of contracture tension, whereas pre-treatment with high-Ca Ringer has an opposite effect. This "priming" with excess K can be substantially altered by shifting the Ca equilibrium of muscle, suggesting that the primary effect may actually involve such a shift.

The K-contracture in turtle muscle is of considerable magnitude: 1.5–2.7 kg./cm.^{2} cross-section area, comparable to that observed in single fibers, suggesting that the diffusion time of K to the extracellular space in this preparation does not limit contracture very greatly.

These observations support the notion that depolarisation as such and by itself does not activate the contractile system, and that depolarisation and activation are not directly linked. Depolarisation might serve as a "priming" step for activation but subsequent events need to be looked for which are more directly linked to activation than is depolarisation.

Calcium, oxytocin and the regulation of the myometrium. E. M. COUTINHO AND A. I. CSAPO.

The notion that Ca is the key ion in the regulation of myometrial function is derived from the observations that Ca-free Krebs increases the threshold of excitation, and decreases tension more drastically in the estrogen than in the progesterone dominated myometrium. The rapidity of the Ca effects suggested alterations in membrane function.

Oxytocin was found to decrease the threshold of excitation, and the promptness of its effect pointed again to an action on the membrane. Further experiments show that "oxytocics" *do not stimulate* a uterus of maximum working capacity if its intact membrane function is temporarily suspended.

Repeated washing in Ca-free Krebs results in a quick and complete loss of myometrial tension. The changes which accompany and explain the loss of tension are: increase in threshold, loss of conduction, and decrease in the duration of the mechanical response (shortening of the active state?). This condition of Ca deficiency is completely reversed in a graded fashion by the gradual increase in the Ca.

The recovery of the Ca-deficient muscle is strongly temperature dependent ($Q_{10} > 3$). Oxytocin completely eliminates the effect of low temperature, a finding which offers the first accurate method for determining minute quantities of oxytocin.

If the uterus is estrogen dominated the drop in tension in Ca-free Krebs is rapid, whereas it is slow if the muscle is progesterone dominated. Maximum tension in the Ca-depleted uterus is obtained at higher [Ca] if the muscle is dominated by estrogen than if dominated by progesterone.

It appears that under progesterone domination Ca is more firmly bound in the myometrial cell or that the cell requires less Ca for tension development than under estrogen domination. Oxytocin seems to be involved in "Ca-transport" or might sensitise the particular structure of the myometrial cell on which the Ca effect is exerted.

Iodide contracture in potassium treated muscle. BRIAN A. CURTIS AND ARPAD I. CSAPO.

When the retractor penis muscle of the turtle *Chrysemys picta* is placed in a modified Ringer containing 50% (60 mM) sodium iodide (replaced for sodium chloride) and 20 mM potassium rather than 2.5 mM, the muscle goes into contracture. No contracture is observed if the iodide or potassium treatments are applied separately. Furthermore if the muscle is first depolarized by high potassium (12–30 mM) and the iodide Ringer is then added, the muscle will go into contracture irrespective of the change, if any, in the potassium concentration within the above range. Thus the iodide has an effect not only if the membrane potential is decreasing, but even if it remains unchanged or is increasing.

If a muscle is depolarized by high (for example 20 mM) potassium, contractures can be repeatedly induced by adding and removing the iodide, keeping the potassium concentration constant.

The effect of the iodide is on the membrane as shown by the rapidity of the onset and cessation of contracture.

These experiments support the general notion that depolarization is a necessary step in the series of events leading to activation. But this step need not be followed by myoplasmic activity, allowing us to keep the muscle in a "primed" condition at rest, subject to later activation. The instantaneous effect of iodide suggests that the step following depolarization is localised to the membrane itself. Further, iodide not only prolongs, but under certain conditions can initiate the active state.

Some aspects of the chemical composition of the aqueous humour and plasma of the smooth dogfish. RUSSELL F. DOOLITTLE, BONNIE BEDFORD, CAROLYN CHESBROUGH, CYNTHIA THOMAS AND WILLIAM STONE, JR.

A comparative chemical analysis of the aqueous humour and blood plasma of the smooth dogfish was undertaken as a prerequisite to further inquiry into the mechanisms of aqueous humour formation and maintenance of the intraocular pressure. The elasmobranch fishes were chosen since the osmoregulation of this class of organisms is very dependent on the retention of great quantities of certain organic compounds, namely, urea and trimethylamine oxide. Inasmuch as such molecules are known to be slow crossing from the plasma to the aqueous in mammals, it was theorized that a large absolute concentration gradient should exist across the so-called "aqueous barrier." Consequently, factors tending to permit any such osmotic differential should be proportionately exaggerated. It was established in this study that a large concentration drop does exist for both urea and trimethylamine oxide. Therefore, in addition to urea and trimethylamine oxide, it was decided to measure certain constituents which were known to be in either excess or deficit in many mammalian aqueous humours. The ascorbic acid content of the aqueous was determined to be higher than in the plasma. Total CO_2 was also much higher, and preliminary pH studies show the pH to be considerably more alkaline. The gross concentration of free amino acids was shown to be much lower in the aqueous than in the plasma. Dry weight determinations were done on both aqueous and plasma, and a rough tabulation of total solids in these fluids was made. Some preliminary measurements on sodium ion concentration were also made.

Some aspects of morphogenesis in ascidians. SYLVIA FITTON JACKSON.

Comparative studies are being made on certain aspects of growth patterns in the ascidians *Ciona intestinalis, Molgula manhattensis, Perophora listeri, Botryllus schlosseri* and *Amaroucium constellatum:* a general survey has been carried out on the main fine-structural characteristics of the different cell types.

In longitudinal sections of the tail of newly shed larvae of *Amaroucium* and *Perophora*, the myofibrils of the myoblasts are well differentiated and the arrangement of the bands is superficially similar to that observed in vertebrate skeletal muscle. The dimensions of most of the sarcomeres seen in electron micrographs are as follows: the length of the sarcimere averages 1.9 μ, that of the A band 1.4 μ, the I band 0.2 μ, the Z line 0.1 μ and the H zone 0.15 μ. The larger filaments of the A band region are each about 230 A wide and are spaced about 200 A apart. The banded structure of adjacent myofibrils is usually in register and the junction between the cells perpendicular to the fibrils axes occurs consistently at the Z line zone, the junction being composed of two outer and two inner dense parallel lines.

Investigations are being made on the mechanism of architecture of the branchial basket, and elaboration of the tunic. The cilia around the periphery of each gill slit are arranged in 7 parallel rows; the cilia are closely apposed to each other and are interconnected at the level of the basal granule by a fibrous component. In regeneration experiments three-day-old zooids of *Amaroucium* have been cut at the base of the branchial basket, the latter has been removed but the surrounding tunic has not been detached. During the next two days regrowth proceeds and tissue forms a stump just above the oesophagus. On the third day primordia of the gill slits lie horizontal to the long axis of the zooid, but by the fifth day four complete rows of slits are present with their long axes parallel to that of the zooid. Calculations indicate that during this period of regeneration a minimum number of 50,000 cilia is reproduced. The subsequent pattern of development of treated zooids appears similar to control zooids which were not cut.

The effect of the ovarian steroids on the membrane potential of the uterus. M. GOTO AND A. I. CSAPO.

Single cells of the rabbit uterus, in well defined endocrine conditions, were impaled with flexible microelectrodes at 25° C. and their membrane potential recorded. The myometrial cell a few days after parturition has a low membrane potential of about 35 mV. Estrogen treatment results in an increase up to 48 mV, whereas progesterone treatment yields values as high as 55 mV.

Between the 20th and 29th day of pregnancy the membrane potential of the uterus at placental implantation sites increases up to 60 mV, whereas the interplacental sites remain at a value of 48 mV. Thus the placenta exerts a local effect in the neighbouring myometrium. Treatment with 5 mg. progesterone/day, between days 20–25, increases the membrane potential of interplacental sites, and the difference between different uterine portions disappears, suggesting that the effective placental product is progesterone.

The membrane potential drops and the difference between different uterine portions disappears after the 29th day, resulting in a uniform potential of about 50 mV at parturition.

The uterine membrane potential is a log function of the K, as in other excitable tissues. In Ca-free Krebs the membrane potential decreases more in estrogen than in progesterone dominated uteri. Action potentials after oxytocin treatment, at 25° C., are synchronous and regular. Local potentials of different frequency appear in the same cell, a finding which can be best explained by intercellular bridges, transmitting the electrical activity of one uterine cell to the next. The myometrial cell membrane, on which excitability and contractility depend, is subject to endocrine regulation. Changes in membrane potential can be well correlated with myometrial function.

Luciferin and luciferase extracts of a fish, Apogon marginatus, and their luminescent cross-reactions with those of a crustacean, Cypridina hilgendorfii.[1] YATA HANEDA, FRANK H. JOHNSON AND EDWARD H.-C. SIE.

Despite numerous attempts to obtain extracts containing the relatively heat-stable substrate, luciferin, and heat-labile enzyme, luciferase, respectively, which react with light emission in aqueous solution (the "luciferin-luciferase reaction"), such extracts have been obtained thus far from less than a dozen of the many types of luminescent organisms known. Moreover, until now, luminescent cross-reactions between these components from different organisms have been found only with extracts of fairly closely related types, such as different families of fireflies or different genera of ostracod Crustacea. Recently (1957), the first clear example of the luciferin-luciferase reaction among fishes was demonstrated, in extracts of *Parapriacanthus beryciformis*, family Pempheridae. A second example is reported herewith, in extracts of either the anterior or posterior photogenic organs of *Apogon marginatus,* family Apogonidae, and these extracts are found to produce light-emitting cross-reactions with partially purified luciferin and luciferase of the ostracod crustacean, *Cypridina hilgendorfii.* Extracts of fresh or desiccated anterior and posterior *Apogon* light organs cross-react with each other, but not with extracts of non-photogenic tissues or of lanterns of the Japanese firefly, *Luciola.*

Quantitative data in cross-reactions between the *Apogon* and *Cypridina* systems reveal that (1) the rate of light emission follows first order kinetics; (2) doubling the enzyme concentration doubles the rate; (3) total light is proportional to initial luciferin concentration; and (4) total light is nearly the same with the luciferase of either organism acting on equal aliquots of luciferin from one organism.

Crude *Apogon* luciferin dissolves poorly in cool water but readily in methanol, giving a yellow solution that fluoresces greenish in ultraviolet. Studies on spectroscopy, chromatography, and possible co-factors of the *Apogon* system are in progress.

Fluorescence, phosphorescence and bioluminescence in the ctenophore, Mnemiopsis leidyi.[2] E. NEWTON HARVEY AND S. P. MARFEY.

The chemical bioluminescent system of Mnemiopsis, located in the radial canals, is unique in several respects. No dissolved oxygen is required for light emission. Bright light inhibits the bioluminescence. No fluorescence of the canals occurs in near ultraviolet light (Keese lamp, 3650 A) in resting Mnemiopsis, but after bioluminescence has occurred the canals become bluish fluorescent, an effect which slowly disappears if the animals remain unstimulated. Such a change from non-fluorescent reductant to fluorescent oxidant occurs among flavins, but we do not imply that a flavin is concerned in Mnemiopsis bioluminescence, as it is in luminous bacteria. The fluorescence along canals is much brighter at acetone-dry ice temperature (ca. − 78° C.), and there is also a phosphorescence, lasting about 3–6 seconds.

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[1] Aided in part by the Office of Naval Research, National Science Foundation, and Eugene Higgens Fund.

[2] Aided by grants from the National Science Foundation and the National Institutes of Health.

If ctenophores are placed in 1% sucrose in sea water and then held at acetone-dry ice temperature, no marked change in phosphorescence was observed. The phosphorescence is diffuse rather than sharply localized in the canal region. The phosphorescence is not connected with the bioluminescent system, because (1) it can be observed in frozen intercanal tissue of Mnemiopsis in sucrose solution, (2) it is observed from frozen mantle and viscera of the non-luminous clam (Mya). Moreover, light-adapted ctenophores, which do not bioluminesce on stimulation, show no fluorescence in radial canals at 23° C. or at − 78° C., but with sucrose added, phosphoresce diffusely at − 78° after near ultraviolet light exposure. Bioluminescent Chaetopterus tissue (rear segments with eggs) showed fluorescence and phosphorescence at − 70° C., whether sucrose was added or not. Far ultraviolet light (Mineralite lamp, 2735 A) does not excite fluorescence or phosphorescence of stimulated Mnemiopsis, with or without sugar, in any region at room temperature, or at − 70° C. Bioluminescence and fluorescence are thus connected, but phosphorescence is apparently a separate phenomenon. Mnemiopsis is recommended as an unusually favorable form for quantitative study of light effects.

Evidence for the splitting-off of S^{35}-labelled sulfate from the fertilizin of Arbacia eggs upon the spontaneous reversal of sperm-agglutination. RALPH R. HATHAWAY[1] AND ALBERT TYLER.[2]

Arbacia fertilizin labelled with S^{35} was obtained as described by Tyler and Hathaway (see separate abstract) and employed in a series of sperm-agglutination experiments. As is well known, the agglutination of sperm by fertilizin in sea urchins reverses spontaneously under ordinary conditions, the duration of agglutination depending upon various factors including concentration of fertilizin, concentration of sperm, and temperature. Under certain conditions (excessive washing and picric acid treatment) reversal does not occur. The present experiments show that when S^{35} fertilizin is absorbed with 20% sperm suspensions under ordinary conditions of agglutination and reversal, very little of the S^{35} is removed from the supernatant by the sperm (13%, 14%, 23%, and 28% in four experiments). On the other hand, removal of S^{35} from supernatants results from absorption with dry sperm for a short time and/or at low temperatures or with sperm treated so as to inhibit spontaneous reversal. For example, in three experiments at room temperatures 72%, 74%, and 64% was removed by dry sperm.

At 0° C. similar experiments gave 95%, 91%, 83%, 83%, 98%, and 72% removed. Simultaneous short absorption experiments, making use of the same batches of dry sperm and fertilizin, showed 81% S^{35} removed from the cold supernatant and 50% removed from that at room temperature. Excessively washed and picric acid treated sperm removed 94% and 99%, respectively.

In these experiments the densities of the mixtures made timing of the duration of agglutination and reversal difficult, but judging from the intervals between mixing and centrifugation it appears that reversal is accompanied by loss of sulfate from fertilizin absorbed to sperm. This implies the presence of an active sulfatase on the sperm and that the phenomenon of spontaneous reversal relates to the liberation of sulfate from fertilizin.

An attempted analysis of schooling behavior in the marine snail Nassarius obsoletus. CHARLES E. JENNER.

Schooling in mud snails in Barnstable Harbor initiates annually as a result of a behavior change associated with the abrupt termination of reproductive activity. This change in be-

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[1] Supported by a National Science Foundation grant to Dr. Charles B. Metz.

[2] Supported by a research grant (C-2302) from the National Cancer Institute, U. S. Public Health Service and by AEC Contract AT(30-1)-1343 to the Marine Biological Laboratory.

havior, marked by greater locomotor activity, results in an aggregated distribution pattern strikingly different from the dispersed pattern found during the reproductive phase. Schooling is most apparent when snails are submerged. With exposure at low tide, many snails within a schooling group will stop schooling and bury in the sand, whereas others within the group will continue to school. High temperature and drying of the substratum, associated with exposure at low tide, are factors which deter schooling. Current plays an important role in determining direction of movement, schooling in general being either into or with the current. However, schooling can be observed on occasion across the current or where there is no current. Physical contact also plays an important role in promoting uniform orientation. Converging snails upon contact will generally display a turning response and then proceed along parallel paths. In dense concentrations of schooling snails, it would be physically impossible for a snail to move in any direction other than that of the group. Vision and chemical factors may also play a role in schooling but this has not yet been demonstrated.

Neural photosensitivity in Mactra. DONALD KENNEDY.

Many pelecypod molluscs which lack obvious photoreceptor structures demonstrate "shadow responses," in which a diminution of light intensity incident upon the siphon causes its withdrawal. The present experiments have revealed, through oscillographic recording from the pallial nerve in *Mactra,* the presence of a single neuron which discharges at high frequency (up to 120/sec.) upon cessation of a light stimulus. Such off-discharges—or the spontaneous activity in the photoreceptor fibers which usually occurs in the dark—are inhibited with short latency by re-illumination. The degree of inhibition is proportional to the intensity of the re-illuminating flash, and the latency of inhibition inversely so. Latency and frequency of the off-discharge itself, however, vary in a complex fashion with respect to the intensity of a single stimulus; the discharge can, in fact, be treated as a post-inhibitory rebound phenomenon.

The discharge may be recorded with unimpaired threshold and pattern in excised segments of the pallial nerve from the siphonal region. It is thus apparent that the primary receptor is a neural element. In *Mya* and *Venus,* off-discharges occur in many fibers of the siphonal nerves, but these originate in end-organs within the siphon. The nerves of *Mactra* (but not of the other two species) contain a pinkish-red pigment. Experiments are in progress to determine whether this pigment endows the receptor neuron with its light sensitivity.

These findings suggest that off-responses do not always arise as a result of inhibitory synaptic interactions between on-responding units, as is clearly the case in several of the more complex photoreceptor systems. Such off-responses as those from the distal cell layer in the mantle eye of *Pecten,* which were presumed by Hartline to be of secondary origin, may in fact prove to be primary.

The humoral control of feeding in Physalia and its evolutionary significance. HOWARD M. LENHOFF AND HOWARD A. SCHNEIDERMAN.

The demonstration of Loomis that reduced glutathione (GSH) induces the feed-response in hydra led us to examine the feeding response of a distantly related hydrozoan, the siphonophore *Physalia physalis* L. During normal feeding, *Physalia* draws up prey to its gastrozooids, the only members of the colony capable of ingesting food. The gastrozooids apply their mouths to the surface of the prey and their lips spread out to envelop it. This "spreading" of the mouths can be elicited in isolated gastrozooids by fish blood. The active principle in the blood appears to be GSH. The sensitivity to GSH is remarkable: 10^{-6} *M* caused 90% of the isolated cylindrical gastrozooids to spread their lips on a glass dish and transform into discs, often more than 20 mm. in diameter. Cysteine did not induce this response. The behavior of isolated gastrozooids in the presence of GSH is identical with the normal feeding behavior of gastrozooids in the intact animal. Hence it appears that in the feeding process of *Physalia,* as in hydra, the nematocysts pierce the prey, thus releasing body fluids containing GSH. This GSH induces the feeding response in the gastrozooids. Consequently, like hydra, *Physalia* only feeds on forms with body fluids.

The chemical similarities of the nematocyst-GSH feeding mechanism of *Physalia* and hydra invite inquiry into evolutionary relations among the Cnidaria. Evidence was presented that a hydrozoan stem-form using the nematocyst-GSH feeding mechanism evolved along with animals having body fluids containing GSH which could be released when pierced by nematocysts; only animals of a higher grade of organization than Cnidaria fit this category. It was also argued that the ancestral cnidarian, from which all Cnidaria arose, probably did not use the nematocyst-GSH feeding mechanism, but was a filter feeder like many present-day Anthozoa.

Genes controlling the movement of flagella in Chlamydomonas. RALPH A. LEWIN.

By genetic analysis of paralyzed mutants of *C. moewusii*, several of the genes controlling the movement of flagella have been located in linkage groups. Two loci, about 9 units apart, are situated on the sex chromosome; one of these is extremely close to the mating-type locus, about 11 units from the centromere. Three mutations have been located at a single locus close to the centromere of a second chromosome. One of these alleles is distinguishable from the others in its degree of paralysis. Two other motility genes are situated on a third and a fourth chromosome, respectively; one is closely linked with its centromere, the other is not.

Following ultra-violet irradiation, apparent reversions of several mutants were isolated. Three behaved genetically like wild-type, indicating reverse mutation at the original locus. One proved completely sterile. A fifth, when crossed with wild-type, segregated irregularly, indicating a suppressor gene or complex.

Fractionation of Cypridina luciferin and its benzoyl derivative.[1] S. P. MARFEY, L. C. CRAIG AND E. N. HARVEY.

Dried, fat-free Cypridina powder was used for isolation of luciferin by extraction with methanol in purified nitrogen at 4° C., and subsequent fractionation by countercurrent distribution, paper chromatography, column chromatography, dialysis, and Reineckate and flavianate precipitation. The best method proved to be countercurrent distribution in purified nitrogen atmosphere at 4° C. employing a variety of acidic solvent systems (containing ascorbic acid which reduces the autoxidation of luciferin) for the separation of two active luciferin fractions. Evidence was obtained from distribution data for the transformation of one of these fractions. The best sample prepared by this method had a chemiluminescent activity with luciferase at least 6000 times (by weight) that of fat-free Cypridina powder and after total acid hydrolysis gave predominantly four amino acids (Lys, Asp, Glu, Ileu) and ammonia together with smaller amounts of several additional amino acids.

Benzoyl luciferin, more stable toward air oxidation, fractionated by dialysis and countercurrent distribution at 25° C. yielded several active fractions. These were analyzed by two dimensional paper chromatography, giving in each case a neutral yellow-colored blue fluorescent area, and after total acid hydrolysis several amino acids (including those found in a larger amount in native luciferin) in addition to an inactive chromophore. Quantitative infrared spectral analysis of these active fractions revealed a variable degree of benzoylation compatible with their polarity in a distribution train. Redistribution of one of these fractions resulted in a single band close to a theoretical curve for one component.

These results give an additional evidence for the chromopeptide nature of luciferin and indicate at least two active types of luciferin separable by countercurrent distribution. The nature of their difference is currently under investigation.

Changes in efflux and influx of potassium upon fertilization in eggs of Arbacia punctulata, measured by use of K^{42}.[2] ALBERTO MONROY[3] AND ALBERT TYLER.

A preliminary set of experiments in 1956 indicated an increase, upon fertilization, in the rate of release of K^{42} from eggs that had been loaded with this isotope. The present series of experiments substantiates this finding and permits closer estimate of the magnitude and the time course of the change. The average values for 25 sets of experiments for ½, 1½,

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[1] Aided by grants from the National Science Foundation and the National Institutes of Health.

[2] Supported by a Research Grant (C-2302) from the National Cancer Institute, U. S. Public Health Service and by AEC Contract AT (30-1)-1343 to the Marine Biological Laboratory.

[3] F. R. Lillie Memorial Fellow, Summer, 1958.

2, 4 and 6 minutes after fertilization are 129%, 154%, 102%, 70% and 58%, respectively, for the excess K^{42} released by fertilized as compared with unfertilized eggs. The corresponding *t*-values for the statistical significance of the difference of these values from zero, are 5.1 ($P < 0.01$), 5.2 ($P < 0.01$), 4.54 ($P < 0.01$), 3.06 ($P < 0.01$) and 1.88 ($P < 0.1$ and > 0.05).

The rate of influx of K^{42} was followed over longer periods of time (up to 6 hours) and found to be considerably greater in the fertilized than in the unfertilized eggs. The calculations show that, at 6 hours, K^{42} in the unfertilized eggs is far below (about 4:1) the estimated equilibrium value (ca. 20:1 for $K_{\text{inside}}/K_{\text{outside}}$), suggesting that much of the K^+ is not freely diffusible. The K^{42} uptake from the fertilized eggs has reached the expected equilibrium value before this time.

In general the rapid change in rate of efflux of K^+ upon fertilization is consistent with the expectation from earlier experiments (Tyler, Monroy, Kao and Grundfest, *Biol. Bull.*, **111**, 1956) demonstrating the existence, in the unfertilized echinoderm egg, of a membrane potential that undergoes a transient drop during the first minute after fertilization and which is reversibly abolished by increase in external K^+.

Action of enzymes on the hyalines of the Arbacia egg. A. K. PARPART, J. CAGLE AND L. WOOD.

The hyaline layer (No. 1) of the egg of *Arbacia punctulata* is formed from cortical granules shortly after fertilization. At first cleavage another layer of hyaline (No. 2) is formed from blebs from the furrow surface. There are therefore at least two hyalines formed.

The action of various enzymes on these two hyaline layers has given the following results. Proteolytic enzymes, trypsin, chymotrypsin and papein have no effect. Lipase also fails to alter these hyaline layers. Amylolytic enzymes act as follows: α-amylase digests and removes hyaline No. 1; it does not alter hyaline No. 2. β-amylase has very slight digestive action on the two types of hyaline. However, it does cause a marked release of hyaline No. 2 from the egg at the time of cleavage. Hyaluronidase loosens but does not digest hyaline No. 1, while it does digest most of hyaline No. 2. The action of the enzymes was studied on eggs whose fertilization membranes did not form due to prior treatment with trypsin.

These and previous studies lead to the conclusion that hyaline No. 1 is a mucopolysaccharide while hyaline No. 2 is composed of hyaluronic acid and/or chondroitin sulfuric acid.

The action of certain chemical agents upon squid chromatophores. WILLIAM ROSENBLUM AND BENJAMIN ZWEIFACH.

The chromatophores of cephalopod molluscs may provide an important tool for studying the basic mechanisms controlling smooth muscle activity. These chromatophores are altered in size by the contraction or relaxation of smooth muscle fibers, each of which is innervated by branches from the "CNS" of the squid. Relaxation of the muscle fibers constricts the chromatophores; contraction expands them.

Substances dissolved in filtered sea water were injected subcutaneously into the squid, *Loligo pealii,* placed alive in sea water below 10 degrees. The following monamines were found to cause local constriction of the chromatophores (relaxation of the muscle fibers): tryptamine HCL, tyramine HCL, and 5-hydroxy-tryptamine creatinine sulfate (serotonin). Serotonin was effective in lower dosage (0.2 μg/ml.) than any of the other substances.

These inhibitors of monamine oxidase also were found to constrict the chromatophores: para isopropyl hydrazine, 2-benzyl-1 picolinyl hydrazine, and iproniazid.

Tryptophan HCL, 5-hydroxy tryptophan HCL, histamine dihydrochloride, L-lysine and L-serine had no effect.

These data are consonant with the theory that chromatophore control at the local level is achieved by the local release of an amine and its continual destruction by amine oxidase.

Other agents which constricted the chromatophores were: LSD-25 which, like serotonin, contains an indole nucleus; chlorpromazine, an autonomic blocking agent; and eserine sulfate, an inhibitor of acetylcholine esterase. The latter is effective in high dosage (1 mg./ml.).

Acetylcholine dilated these chromatophores. Serotonin reversed this. It may be that chlorpromazine acts by blocking the action of a naturally occurring dilating agent, such as acetylcholine, and thereby allows the constricting agent to act unopposed. The effect of eserine may be attributed, as in the case of paralysis of other nerve-muscle systems by eserine, to interference with the cholinergic mechanism.

It may be significant that *all* the constricting substances contain a resonating moiety.

(This work was made possible by a grant to one of us—W. R.—from The United States Public Health Service.)

Contraction without membrane potential change. T. SAKAI AND A. I. CSAPO.

It had been shown in our laboratory that muscles, rendered non-propagating by treatment with procaine or substitution of Na with choline, in excess K Ringer, contract along their entire length (except the extreme anodal portion) when stimulated in a longitudinal field. It is of considerable significance to determine whether activation of the myoplasm, in resting muscle pre-treated with excess K, requires depolarisation of the excitable membrane or that currents flowing inside the muscle fibers, as a result of the applied longitudinal electric field, can accomplish this task. We have measured, therefore, with microelectrodes the membrane potential changes along the length of the muscle during the application of the longitudinal electric field, under similar conditions in which shortening has been previously determined. We found that the membrane potential is unchanged in the middle portion and is increased in the anodal half of the muscle where shortening does take place. Thus the longitudinal field can activate a K depolarised resting muscle without change or even with slight increase in membrane potential. Since the "priming" step, accomplished by K treatment, leaves behind a resting muscle, it is justified to conclude that steps subsequent to depolarisation are required to complete the coupling process. These then are more directly linked to activation than is depolarisation. Currents, which are known to flow inside the muscle fibers during normal excitation, may well contribute to one of these steps.

The unique significance assigned to depolarisation in the activation process is also challenged by the experiment in which the fiber membrane was removed by micro-surgery and the "naked" myofibrils were activated, under oil, by longitudinal current. The threshold current required for the activation of naked myofibrils is of the same order of magnitude as the myoplasmic currents of normal excitation.

Antibacterial action of Limulus blood in an in vitro system. MANOHAR V. SHIRODKAR, FREDERIK B. BANG AND ANNE WARWICK.

An "intact system" was developed based on the previous findings of Bang and Warwick. Two cc. of Limulus blood in a siliconized syringe and needle were explanted sterilely into a siliconized roller tissue culture tube and kept in a rotating drum at room temperature. A high percentage of amoebocytes remained intact in shape and granule content for over 30 days without addition of nutrient medium. No antibiotics were used. Two such tubes were kept unstoppered for a month without showing bacterial contamination or major cytological changes. A "partially intact" system was similar in other respects except that no silicone was used and the majority of cells lost granules and changed to a flattened form. Use of the intact and partially intact systems was made in demonstrating the potent antibacterial action of Limulus blood. Sterile artificial sea water without sodium bicarbonate was used for dilutions and samples were plated on ZoBell's sea water agar. A number of timed, quantitative experiments were performed with bacterium No. 5, a gram-negative, motile rod isolated from oysters. Within 6 hours' incubation at room temperature in the intact system, 24,000,000 bacteria were eliminated, no viable bacterium being recoverable even after 120 hours. The partially intact system showed some antibacterial activity up to 24 hours, after which the bacteria reappeared and grew successfully. Other experiments showed that an intact system could not inhibit growth of some bacteria except, perhaps, at very high dilutions. Good to intermediate antibacterial activity was demonstrable against 6 of the 8 different bacteria tested in the intact system. Current experiments have thus far failed to show antibacterial activity in the serum fraction, alone, of Limulus blood.

Amino acid uptake in marine invertebrates. G. C. STEPHENS AND R. A. SCHINSKE.

These observations were carried out to extend our previous report of the uptake of amino acids from sea water by ciliary-mucoid filter feeding animals. Thirty-five species, representing

12 animal phyla, were used. Glycine was used in all cases; sometimes additional amino acids, such as alanine, methionine, glutamic acid, arginine, phenylalanine and tyrosine were employed. After placing an animal in 2 mM amino acid, periodic samples of the solution showed a quantitative decrease of the acid when measured by means of a ninhydrin colorimetric technique.

The addition of suitable concentrations of penicillin, streptomycin or tetracycline had no apparent effect on the uptake of amino acid. This, together with the negative results for arthropods, indicates that bacteria are not responsible for amino acid removal. Such removal also occurs from artificial sea water. Finally, it was possible to demonstrate directly a 3%–8% drop in amino acid concentration when water collected from the excurrent siphon of the clam *Spisula,* was compared with that collected from the incurrent siphon.

The concentration of amino acids used in most of these observations was approximately two orders of magnitude above estimated content of organic material in naturally occurring sea water. Consequently experiments were done using 0.02 mM amino acid solutions. By means of concentration and subsequent acetone-HCl extraction, uptake at these levels could be detected. The time for total clearance in those cases where it was observed was not strikingly different despite this hundred-fold difference in concentration.

No effort was made to confine attention to ciliary-mucoid filter feeders. Several detritus feeders and large particle feeders were included among the species manifesting this capacity for amino acid removal.

Synthesis of ribonucleic acid by nucleoli. W. S. VINCENT, B. BENSAM AND ARLENE BENSAM.

Although the nucleolus is known to contain RNA there has been no satisfactory demonstration that it actually synthesizes this material. In the experiments described below we present evidence which indicates that the starfish oocyte nucleolus either synthesizes or accumulates newly synthesized RNA.

If cells are incubated in the presence of a supply of radiophosphate, the pools of 5′ nucleotides will be labelled and subsequently incorporated into the RNA polynucleotide chain. If synthesis of RNA occurs, isolation and hydrolysis of the RNA, followed by separation of the nucleotides, will reveal the presence of radiophosphate in all four of the constituent nucleotides. Lack of label in nucleotides is indicative of no new synthesis of RNA.

When starfish ovaries were incubated in sea water containing P^{32} for 1½ hours, the RNA from the nucleoli contained considerable radioactivity which was tightly bound to the polynucleotide. After hydrolysis by alkali and separation of the nucleotides by paper electrophoresis no radioactivity could be detected in any of the nucleotides. All of the label formerly associated with the RNA was found as inorganic phosphate. Other experiments revealed that this phosphate could not be removed from the RNA by 7-minute hydrolysis in acid, indicating that it was not bound by a pyrophosphate linkage.

When incubation time was extended to 6 hours all of the nucleotides were found to contain the radioisotope, indicating the presence of newly synthesized RNA. Considerable amounts of the additively bound phosphate found in the 1½-hour experiments were still present.

These experiments are interpreted as demonstrating an initial binding by nucleolar RNA of phosphate entering the nucleolus. Subsequently either this bound phosphate, or phosphate from other sources, is found in newly synthesized RNA in the nucleolus.

PAPERS READ BY TITLE

Methyl green "vital staining" in Arbacia eggs. WALTER AUCLAIR.

E. B. Harvey reported that methyl green gives a distinctive purple stain to the mitochondria of centrifuged unfertilized eggs and this has been confirmed in the present experiments. In addition, it was found that single spherical granules situated each in one of the echinochrome vesicles, and each having a diameter about ⅙ that of the vesicle, take an intense blue-black color when eggs are placed in methyl green-sea water solutions (1:10,000) for 5–10 minutes.

Such stained granules are also found in uncentrifuged eggs, both fertilized and unfertilized, and in fertilized eggs centrifuged with and without pressure (6000 lbs./in.^{2}) at 41,000 × gravity. After a thorough washing of the eggs in sea water, the staining persists throughout subsequent development. Equivalent staining of the granules in the pigment vesicles takes less time (3–5 minutes) after centrifugation than without centrifugation (8–10 minutes).

Seen at high magnification (900 ×), the movement of these methyl green-stained granules appears to be distinctive. Each maintains continuous contact with the wall of the pigment vesicle in which it lies and yet all the granules continually display a sort of dancing movement. This implies that each vesicle may display a haphazard sort of rotational movement, but this tends to diminish as the successive cleavages ensue. In unfertilized eggs the echinochrome vesicles display sudden, short, straight-line excursions in addition to the rotational movement.

Cleavage of stained eggs is somewhat abnormal. Often the first cleavage is aborted and two or even three nuclei may be seen in the single cell. At the time when second cleavage normally occurs, many of the stained eggs divide into two, or, more frequently, into three blastomeres. Subsequently the blastomeres are apt to be of different sizes, and tend not to adhere together, but about 30 per cent develop to swimming blastulae (mainly abnormal).

Antibacterial activity of Phascolosoma govldii blood.[1] F. B. BANG AND S. M. KRASSNER.

Blood removed from the body cavity of normal *Phascotosoma gouldii*, a sipunclid worm, was found to be sterile when cultured on ZoBell's sea water agar at room temperature.

Several strains of marine bacteria were destroyed within twenty-four hours when more than 100 million organisms were injected. An occasional infection was produced which eventually killed the *Phascotosoma*. Blood removed from the worms was incubated at room temperature with varying concentrations of different bacteria and in a number of individual tests became sterile within six to twenty-four hours. Consistent sterility was obtained within six to twenty-four hours when the combination of bacteria and blood was kept at 0° C. Various control preparations kept these bacteria alive for days at this temperature. At 0° C. incubation activity was found in both serum and cells. Destruction of 1 million organisms was obtained with 0.2 cc. of whole blood within twenty-four hours. Sera and bacteria kept at 0° C. failed to agglutinate.

Enrichment studies on the photosynthetic sulfur bacteria. EDWIN H. BATTLEY.

Although several genera of the Thiorhodaceae and Chlorobacteriaceae have been recognized, largely on the basis of a morphological classification, extensive studies of these organisms have been hindered by a lack of pure cultures. This lack may possibly exist because classical methods of selectively isolating these bacteria from their natural habitat most frequently give rise to organisms of the *Chlorobium* or of the *Chromatium* type. For this reason it was thought worth while to search for methods of selectively growing other types of photosynthetic sulfur bacteria. An inspection of their marine habitats made it appear that these bacteria grew most abundantly in the presence of decomposing plant or animal matter. This made it seem that classical enrichment media, supplemented with vitamins, trace elements and various organic carbon donors, might select differently. Thus, a basic medium containing the usual amounts of phosphate, carbonate, sulfate, ammonia nitrogen, potassium, magnesium, calcium and iron was used, plus 2.4 per cent sodium chloride. Zinc, boron, cobalt, copper and manganese were added as trace elements, and biotin, calcium pantothenate, inositol, nicotinamide, para-amino benzoic acid, thiamin hydrochloride and riboflavin as vitamins. Specific media were made by adding to the basic medium 0.4 per cent sodium sulfide or 1.0 per cent sodium thiosulfate, and 0.1 per cent sodium acetate, 0.05 per cent peptone or 0.05 per cent yeast extract. The specific media were adjusted to pH 7.0 or 8.0. Mixtures were made of the specific media and samples of marine mud. Glass-stoppered bottles were then filled with the mixtures and placed at room temperature under strong illumination. Abundant growth usually occurred within three or four days. After the third transfer predominant organisms were present to

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[1] Supported by a grant-in-aid from the National Institutes of Health.

the extent of 90 per cent or better. About half of these organisms have not been previously described.

A Bermudian marine Vaucheria at Cape Cod. A. J. BERNATOWICZ.

Vaucheria nasuta Taylor and Bernatowicz, heretofore known only from Bermuda, is reported from the salt marsh at Barnstable, Massachusetts. With this record the number of marine Vaucherias known from northeastern North America is raised to ten, of which seven occur on Cape Cod. Records of the earlier finds suggested that reproduction in local Vaucherias may be confined to the winter months, but the present material was reproductive, although sparsely, in August.

An inhibitory extract of chick tissues. MAXWELL H. BRAVERMAN.

The imposition of extracts of adult tissues upon embryonic systems has proved an excellent tool for investigating the order of tissue differentiation and mode of tissue interaction. The organ or organ part to be used in the test is cut from a freshly-killed animal and immediately put into Tyrode's solution (adjusted to pH 7.4–7.6) at 0° C. All subsequent operations are carried out at this temperature. The tissue is homogenized in a tissue homogenizer by hand or in a test tube fitted with a plastic plunger which is mechanically rotated. The homogenate is centrifuged at $5000 \times g$ in a pre-cooled centrifuge and the supernatant filtered through a Seitz filter under pressure. The clear liquid is stored for the two or three hours between preparation and utilization in injection bottles which hold 10 cc.

The bottles are heated immediately before use to about 35° C. by placing them in small water-filled beakers on a slide warmer. Two-tenths cc. of the liquid is removed into a 0.25-cc. syringe previously sterilized at 160° C. for 40 minutes in an autoclave. The solution is slowly injected beneath the blastoderm of an egg which has been incubating for 24 hours. To make the injection, a square window is sawed in the shell with an "Xacto" No. 34 "razor saw" and a small amount of albumin removed by means of oral suction, into a glass pipette. An attempt is made to put the needle in as closely parallel to the egg surface as possible, thus liberating the fluid close to the blastoderm. The hole is sealed with Scotch tape.

The eggs, after 24 hours' incubation at 37.5° C. are in Lillie's stage 4 or 5. The injected eggs are examined from 24 hours to five days after injection.

The explanation of the two-day physiological anticipation of barometric pressure changes.[1] FRANK A. BROWN, JR. AND FRANKLIN H. BARNWELL.

It has been reported that a wide variety of animal and plant species exhibit, notably in their 5–6–7 PM metabolic rate, a lead correlation with the mean daily barometric pressure of the second day thereafter. This they do even when maintained in constant conditions, including pressure. The mean lead correlations have been reported to range from $r = .27$ to $r = .84$ with an average of about 0.5. In attempting to account for this extraordinary capacity of living things it was recalled that the 6 PM metabolic rate had been found correlated with the concurrent 2–6 PM rate of barometric pressure change. It was now found that the 2–6 PM rate of pressure change itself, in Chicago, during each of the years 1954 to 1957, while showing little or no correlation with the mean pressure of the same day ($r = -.14$), was showing a maximum correlation ($r = +.35$) with the pressure of the second day thereafter. The form of the lag-lead correlation-relationships between these two pressure parameters was found to resemble very closely the form of the published lag-lead correlation relationships between mean pressure and the 6 PM metabolic rate in organisms as diverse as fiddler crabs and potatoes. From this, it is concluded that a direct response of the organism to some pervasive external force correlated with the 2–6 PM rate of pressure change comprises the means of the reported organismic anticipation of the pressure changes.

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[1] These studies were aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

The rhythmic nature of metabolism in Ilyanassa in constant conditions.[1] FRANK A. BROWN, JR., WILLIAM J. BRETT AND H. MARGUERITE WEBB.

Hourly values of O_2-consumption of the mud snail, Ilyanassa, were obtained continuously for 33 days under conditions of constant temperature, pressure and illumination. On no day were less than 16 nor more than 48 snails involved. During this study there was found to be a highly significant solar-day cycle (9.7%) with a major maximum about midnight and a lesser maximum about noon. There was a lunar-day cycle (6.6%) with a broad maximum centered on lunar zenith and a lesser maximum at nadir. Mean daily rates of O_2-consumption exhibited a lunar monthly cycle with maximum (first to sixth day after full moon) being 45% greater than for the minimum (third to eighth day after new moon) ($p < 0.001$). Also the 5–7 AM values of O_2-consumption (as deviations without sign from the daily mean) correlated with the concurrent daily 2–6 AM mean barometric pressure change; $r = 0.587$, $N = 33$, $t = 5.0$. There was also a highly significant correlation, but of a different character, between the 2–6 PM mean pressure change and the 5–7 PM deviation in O_2-consumption from the daily mean. There were noted to be striking similarities between the forms of the mean solar- and lunar-day cycles, of *Ilyanassa* and the forms of the mean cycles for the sea-weed, *Fucus,* similarly obtained under constant conditions.

Correlation between O_2-Consumption in Fucus in constant conditions, including pressure, and specific barometric-pressure parameters.[1] FRANK A. BROWN, JR., H. MARGUERITE WEBB AND WILLIAM J. BRETT.

Oxygen-consumption was monitored for about one lunar month during each of the five summers, 1954 through 1958, in *Fucus* kept in constant conditions of temperature, light, pressure, and humidity. A total of 130 uninterrupted days of data was obtained. Highly significant solar-day (amplitude 5.8%; two maxima, 4 AM and 10 AM) and lunar-day (amplitude 4.6%; major maximum about zenith + 6 hours, major minimum about nadir + 6 hours) cycles, and consequent synodic monthly ones were found. Reducing concurrent barometric pressure and *Fucus* O_2-consumption data for the five-year period by averaging all data obtained on each of 30 corresponding days re: new moon and full moon, a correlation was found between the mean 2–6 AM rate of barometric pressure change (without sign) and the concurrent daily 3–5 AM percentage deviation in O_2-consumption (with sign) from the daily mean rate; $r = 0.67$, $N = 30$, $t = 6.5$. There was also a correlation between the average 2–6 PM rate of barometric pressure change (with sign) and the mean 5–7 PM percentage deviation in O_2-consumption (with sign); $r = 0.65$, $N = 30$, $t = 6.1$. Since the barometric pressure parameters are essentially random in their day-to-day fluctuations, it is concluded that the cycle-periods, and, in part, cycle-form as well, are exogenous in *Fucus.*

The interrenal of the sting ray. K. A. BROWNELL AND F. A. HARTMAN.

The interrenal tissue of the sting ray (*Dasyatis centrura*) is usually limited to a single organ, shaped like a thick handled dumb-bell. It lies posteriorly between the kidneys, slightly embedded in the renal tissue on the right. Rarely, small isolated islets of interrenal tissue may be found in the same neighborhood. The gland can be found best by its shape and location since its color is so nearly that of the kidney, due to the thick-walled capsule which covers the characteristic cream-colored tissue. When considered in relation to body size, the sting ray has one of the smallest interrenals among the elasmobranchs. Fifteen males possessed glands 0.00057 ± 0.000051 per cent of the body while twenty-two females possessed glands 0.00070 ± 0.00026 (standard error) per cent of the body.

For histological study the capsule should be removed to permit more rapid penetration of the fixative. The gland is composed of rather small cells without distinct zonation. These cells are arranged in clusters of various sizes and shapes. The nuclei are spherical and

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[1] These studies were aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

irregularly located in the cell. In some specimens, cells of the peripheral region contain more cytoplasm than those more centrally located.

Chromosomes of the estuarine isopod, Cyathura sp. W. D. BURBANCK AND MADELINE P. BURBANCK.[1]

Specimens of *Cyathura* sp. were collected for cytological study in May and June of 1958 from Pocasset River which empties into Buzzards Bay about fifteen miles north of Woods Hole, Mass. These isopods are abundant along the edge of the channel in the upper reaches of the marsh and were the subject of an intensive ecological study from September, 1957 to September, 1958. To obtain gonadal material, the animals were beheaded and the contents of the body cavity dissected out onto a coverslip. Several drops of Nissenbaum's (10:2:2:5 of mercuric chloride, acetic acid, formalin and tertiary butyl alcohol) followed by acetic alcohol, 95% and 100% alcohols and a colloidin-ether solution fixed the material and affixed it to the coverslip. Staining was done in warm Gomori's haematoxylin. Female cyathuras collected in May contained eggs, but it was difficult to retain the material on the coverslips, and what eggs remained affixed, contained no discernibly stained nuclei. Males collected in May and June contained mature sperm and sperm in various stages of development. In some testes there were prophase stages of what appeared to be the first maturation division and in the vasa deferentia of the same animal were sperm with long tails. Usually each testis contained several stages of spermatogenesis. Chromosome counts were made of bivalents at metaphase of the first maturation division and of metaphase and anaphase chromosomes of the second maturation division. The n number of chromosomes is 5. The chromosomes are small, ranging from less than 1 μ at Telophase II to Metaphase I bivalents about 3 μ long. One of the five is much smaller than the others.

Observations on the structure of the cercaria of Himasthla quissetensis. ROBERT R. CARDELL, JR. AND DELBERT E. PHILPOTT.

Cercaria were obtained in the free swimming form, fixed in 1% osmic acid, and electron microscopic studies carried out on the structure of the tail. Incidental to the study of the tail, observations were made on the spines, bacilliform rods and cuticle.

The cuticle of the tail was found to be one micron thick, possessing an outer single membrane with many indentations and a double basement membrane. Small mitochondria were found scattered throughout the cuticle but were concentrated near the smooth muscle layer, found immediately below the basement membrane. The smooth muscle appeared as concentric bands around the tail, approximately 0.5 micron in thickness. A layer of striated muscle approximately three micra thick was found below the smooth muscle, and was directed obliquely to the long axis of the tail. A study of the muscle did not reveal the characteristic A and I bands and distance between the Z membranes was 0.8 micron. The muscle tissue did not appear to enter the body of the cercaria. Further investigation, however, is necessary to substantiate this observation. Mitochondria were concentrated in a band just below the striated muscle whereas the nuclei were located in the center of the tail.

The cuticle of the body was found to be four micra thick with an outer membrane and a basement membrane. In the cuticle structure were found many unidentified spherical to oblong osmiophilic structures. The bacilliform rods were 0.4 micron in diameter, 2.3 micra in length, with a canal through the center and concentric lines around the canal in cross section. The spines, which were embedded in the cuticle, were found to be triangular in cross section with an altitude of 0.4 micron. The base of the spines rested above a thickened basement membrane.

Regulation of flashing in the firefly. JAMES CASE AND JOHN BUCK.

The debated question of whether firefly luminescence is controlled by the nervous system was settled by demonstrating with *Photuris versicolor* that a characteristic neural volley recorded at the terminal ganglion of the ventral nerve cord precedes each spontaneous flash by 60 to 70 msecs. Upon electrical stimulation the neuro-photogenic system exhibits facilitation, treppe, tetany, and has strength-duration relations similar to those of arthropod nerve-muscle. Temperature coefficients for flash latency in the isolated organ are 2.7 at 10° C., 2.2 at 20° C., and 2.0 at 30° C. Intact fireflies differ from decapitated specimens in exhibiting faster cord transit rate, 0.66 m/sec. *versus* 0.09 m/sec., as well as lower threshold.

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[1] Supported by the McCandless Fund of Emory University and an Office of Naval Research-Marine Biological Laboratory Institutional Grant.

These and other measurements indicate that the neural element of the neuro-photogenic system is similar to that of the insect neurò-muscular apparatus. However, the behavior of the effector element differs from that of muscle in a number of ways. Response latency of photogeny to presumably direct stimulation is 9 msec., longer than in either *Mnemiopsis* or polynoid photogeny and much longer than in striated muscle. In addition to flashing, the organ seems able to produce a long lasting glow which can be enhanced, or depressed under certain conditions, by repetitive stimulation both in the presence and absence of flashing. Masking experiments show that the multiple flashes characteristic of some species may be given by as little as five per cent of the total lantern area and hence are not necessarily due to different segmental organs or populations of photogenic cells lighting in relays. However, the organization of the lantern does permit, on occasion, the luminescence of different areas of photogenic tissue independently and in varying sequence.

A source of the toxic factor(s) in scalded starfish.[1] ALFRED B. CHAET AND STAFFORD I. COHEN.

The significance of the toxic factor theory in heat death of invertebrates (*Phascolosoma gouldii* and *Asterias forbesi*) has been previously demonstrated (Chaet, 1951, 1955, 1956). The present report deals with the origin of the toxin released from scalded starfish which causes autotomy and eventual death when injected into normal recipient Asterias.

Starfish were dissected into seven fractions: central discs, lateral portion of ray, aboral portion of ray (including tube feet), hepatic caeca, gonads and coelomic fluid. These fractions were then suspended in sea water (except in the case of coelomic fluid) and heated in non-toxic bags for 1½ minutes at 76° C. Injections (0.15 ml./gm.) into recipient starfish showed all fractions, except the coelomic fluid, to contain a heat-stable, dialyzable toxin similar to that obtained from *in vivo* scalded starfish. It is interesting to note that although the cells of the coelomic fluid proved to be the source of the toxic factor in *Phascolosoma gouldii*, the cell-containing coelomic fluid of starfish did not release any toxin when heated *in vitro*.

In a search for tissue common to all six fractions yielding the toxic factor, two tissues were analyzed; namely, nerve and epithelial. Non-toxic extracts were obtained from radial nerves which had been heated *in vitro*. On the other hand, when the layer of epithelium which lines the perivisceral cavity was isolated and heated, a toxin was in fact released. The physical properties of the "epithelial toxin," as well as its biological activity, have been measured. Like the toxin found in scalded starfish, it is a heat-stable, dialyzable substance.

Survival of Uca pugnax in sand, water and vegetation contaminated with 2,4 dichlorophenoxyacetic acid. C. LLOYD CLAFF, FREDERICK N. SUDAK AND VALERIE MOLONEY.

Fiddler crabs (*Uca pugnax*) were exposed to various concentrations of the commercial weed killer 2,4 dichlorophenoxyacetic acid which was sprinkled on the sand and vegetation in their confinement basins. In a series of animals which were exposed continuously to concentrations of 10,000, 5000, 2500, and 1000 p.p.m. (recommended spray concentration), "2,4-D" was 100% lethal after 108 hours' exposure to 10,000 p.p.m. (50% dead in 72 hours) and 5000 p.p.m. (50% dead in 96 hours), after 10 days in 2500 p.p.m. and 14 days in 1000 p.p.m.

Another series of animals were exposed to "2,4-D" for 12 hours, rinsed in fresh sea water, and placed in confinement basins containing fresh sea water, sand and vegetation. Fifty per cent of the animals exposed to 10,000 p.p.m. were dead in 72 hours after they were placed in uncontaminated basins; 80% were dead after two weeks. A single 12-hour exposure to recommended spray concentration (1000 p.p.m.) was lethal for 20% of the animals within two weeks.

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[1] This study was supported by a grant from the National Science Foundation.

A study of ribonucleic acid during the development of Ilyanassa obsoleta.[1] J. R. COLLIER.

A micromethod based on the procedure of Ogur and Rosen was used for separation of the ribonucleic acid (RNA) in the *Ilyanassa* egg, and the amount of RNA was determined by spectrophotometry.

The fertilized egg contains 0.0032 gamma of RNA. The yolk platelets were separated from the cytoplasm by low speed centrifugation and all of the RNA was recovered in the cytoplasmic fraction.

By the end of the third day of development, at 20° C., there had occurred a three-fold increase in the RNA content of the embryo. After this stage the RNA content continues to increase only slightly. No determinations were made on the fully differentiated veliger.

The RNA content of the first two blastomeres was determined, and the AB blastomere was found to contain 0.0015 ± 0.0001 gamma and the CD blastomere 0.0019 ± 0.0001 gamma of RNA. The sum of the RNA found in the two blastomeres represents a recovery of 106.2 per cent of the RNA of the egg. The AB and CD blastomeres receive 46.8 and 59.3 per cent, respectively, of the RNA of the whole egg. The distribution of the volume of the hyaline protoplasm to the first two blastomeres is: AB, 42.1 per cent; CD, 57.8 per cent. Thus, if the volume of the hyaline protoplasm is used as a reference unit, these data indicate that the RNA is not concentrated in either of the first two blastomeres. Using the dipeptidase activity, or protein content of the egg and blastomeres as a reference unit, there appears to be a slight concentration of RNA in the AB blastomere.

Some characterization of the egg membrane lytic agent derived from sperm extracts of Hydroides hexagonus.[2] ARTHUR L. COLWIN AND LAURA HUNTER COLWIN.

It has been shown (Colwin and Colwin, 1958a) that an extract prepared from frozen-thawed sperm can dissolve the principal component of the egg membrane, *viz.*, the thick *middle layer*, but fails to attack the much thinner *outer border layer* and *inner border layer*. To characterize the lytic agent the following measures were taken. Frozen-thawed sperm was ground with sand in sea water and centrifuged for 90 minutes at 25,000 × g in the refrigerated Spinco. The resultant supernatant had strong lytic activity and completely dissolved the thick middle layer. The supernatant was inactivated if treated with solutions of crystalline trypsin or crystalline chymotrypsin; in each case a final concentration of 0.177% was used and the pH was adjusted to that of sea water. In control eggs, treated with these enzyme preparations, the thick middle layer was not affected. If the trypsin was first treated with soy bean trypsin inhibitor and then added to the sperm supernatant, there was no loss of lytic activity. The active lytic agent in the supernatant is non-dialyzable but inactivated when boiled. Saturation of the supernatant to various degrees with $(NH_4)_2SO_4$ gave the following results: 25% saturation produced a brownish green precipitate with lytic activity. After removal of this precipitate, additional solid $(NH_4)_2SO_4$ was added to bring the supernatant to about 60% saturation; this treatment produced a white precipitate, also with lytic activity. After removal of this second precipitate further solid $(NH_4)_2SO_4$ was added to complete the saturation of the remaining supernatant; this produced a very small amount of white precipitate with little or no lytic activity and a supernatant which was completely inactive. All these precipitates as well as the final supernatant were dialyzed against sea water before testing.

From the above observations it is concluded that the lytic agent in the sperm extract is probably a protein, presumably of an enzymatic nature.

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[1] Supported in part by a grant (A-1899) from the National Institutes of Health, U. S. Public Health Service.

[2] Supported by a Grant (RG-4948) from the National Institutes of Health, U. S. Public Health Service.

The effects of certain enzymes and other substances on the egg membranes of Hydroides hexagonus.[1] LAURA HUNTER COLWIN AND ARTHUR L. COLWIN.

During a study of sperm entry observations were made of the ways in which various substances affected the "vitelline membrane." Eggs immersed in test solutions were examined under the light microscope. A sperm extract (Colwin and Colwin, 1958) dissolves the thick *middle layer* of the membrane without destroying the thinner *outer* and *inner border layers.*

In the concentrations used, hyaluronidase dissolved the middle layer, appeared to separate certain elements of the outer border layer and may have weakend the inner border layer in some way. Trypsin and chymotrypsin had no apparent effect on the middle and inner layers but seemed to cause slight fraying of the outer border layer. Alpha-amylase had no apparent effect on any part of the membrane, either when used directly, or following previous treatment of the eggs with trypsin, chymotrypsin or sperm extract, followed by rinsing in sea water.

Protamine sulfate and digitonin did not dissolve any layer of the membrane. Sodium lauryl sulfate may have modified the outer border layer but did not dissolve the middle or inner layers.

In glucose, glycerol, urea, sodium chloride, and distilled water the middle layer swelled and disappeared. If sea water was added before the dissipation became complete, part or all of the middle layer material reappeared, sometimes widely dispersed. The outer border layer expanded like a balloon and usually broke and was shed; in some solutions its remnant persisted, in others it seemed to vanish. The inner border layer disappeared in sodium chloride and in glucose, and became weaker or thinner in some of the other solutions. More detailed studies, probably by electron microscopy, are needed to clarify the changes in the inner and outer border layers. Sea water at pH ranging from 5.7 to 8.5 did not appear to destroy any of the three layers of the membrane.

Throughout these experiments the same results were observed in fertilized and in unfertilized eggs.

Membrane removal from the egg of the annelid, Hydroides.[2] DONALD P. COSTELLO.

During a comparative study of the membranes of marine invertebrate eggs, it was found that the eggs of *Hydroides hexagonus* could be divested of their vitelline membranes. If unfertilized eggs are treated with successive changes of isosmotic NaCl brought to a pH of 10.5 by the addition of Na_2CO_3 (Costello, 1945), the membranes become sticky, the eggs clump together, the vitelline membranes elevate (at first irregularly and asymmetrically), and, with gentle agitation, the egg membranes rupture or dissolve to permit the eggs to roll out. The membranes themselves disappear (dissolve) except where a large egg clump was present. More than one change of the alkaline NaCl is required to eliminate the divalent cations of sea water, on which the stability of the membrane depends. During treatment, the germinal vesicles break down (indicating parthenogenetic activation) and later the polar bodies are extruded; some of the denuded eggs cleave. A plasma membrane is present on the surface of the denuded egg, and this becomes crenated on further exposure to alkaline NaCl, just prior to eventual cytolysis. The action of the alkaline NaCl can be partially reversed in its early stages, or stopped at any stage, by returning the eggs to sea water. The vitelline membranes of fertilized eggs may be removed in essentially the same manner.

Hydroides egg membranes do not dissolve in 1% Duponal (sodium lauryl sulphate) in sea water (or in NaCl), as does the Nereis egg membrane (Osterhout, 1950). Duponal-treatment demonstrates that the vitelline membrane consists of two layers, and that there are numerous weak spots (microvilli?), through which materials from within the egg may be forced out.

Experiments on the development of fragments and isolated blastomeres of the Hydroides egg are now feasible and will be undertaken.

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[1] Supported by a Grant (RG-4948) from the National Institutes of Health, U. S. Public Health Service.

[2] Aided by a grant (RG-5328) from the National Institutes of Health.

The action of substituted phenols on the conversion of glucose-1-C^{14} and glucose-6-C^{14} to carbon dioxide by the eggs of Arbacia punctulata. ROBERT K. CRANE, A. K. KELTCH, C. P. WALTERS AND G. H. A. CLOWES.

In confirmation and extension of previous work, it has been found that the absolute and relative rates of CO_2 formation from glucose-1-C^{14} and glucose-6-C^{14} by 24-hour embryos of Arbacia can be altered by low concentrations of a series of substituted phenols. Seven representative compounds were tested and it was found that they could be divided into three groups according to their pattern of action. In the absence of phenol the rate of CO_2 formation from glucose-1-C^{14} was 2–3 times the rate from glucose-6-C^{14}. The first group (2,4-dinitrophenol, 4,6-dinitro-o-cresol, 2,4-dinitro-4-chlorophenol, and 2,4-dichlorophenol) was characterized by stimulation of the rate of CO_2 formation from both compounds and an enhancement of the rate from glucose-6-C^{14} relative to that from glucose-1-C^{14}. The rates from both compounds were maximally stimulated by the same phenol concentration and these stimulated rates were approximately equal to each other. The second group (2,4,5-trichlorophenol and pentachlorophenol) also produced stimulation of CO_2 formation from both glucose-1-C^{14} and glucose-6-C^{14}. However, this group did not enhance the relative rate from glucose-6-C^{14} to the same extent: at maximal stimulation, CO_2 formation from glucose-1-C^{14} still exceeded that from glucose-6-C^{14} by 50 per cent of the control amount. The third pattern of action was exhibited by 2,4-dinitrothymol, which did not stimulate CO_2 formation at any concentration tested. On the contrary, at a concentration of 1.6×10^{-5} *M*, dinitrothymol reduced CO_2 production from glucose-1-C^{14} to 60 per cent of the control value and that from glucose-6-C^{14} to less than 4 per cent. The first two groups appear to inhibit, although to different degrees, the phosphogluconate pathway of glucose utilization. Dinitrothymol, on the other hand, appears to exert a heretofore unsuspected profound inhibition on the glycolytic pathway. Experiments on the site, as well as the mode of action, of these substituted phenols are projected.

Substrate induction of adenosine deaminase activity in Arbacia embryos. DAVID DUBNAU.[1]

Enzyme induction may furnish a valuable tool with which to study the synthesis of specific proteins in developing tissues. The induction of enzymes by their substrates has also been utilized in the formulation of a number of hypotheses concerning the mechanism of cellular differentiation. A substrate-induced increase of adenosine deaminase activity has been demonstrated in *Arbacia punctulata* embryos and is reported below. Sufficient adenosine to provide a final concentration of 0.1 mg./cc. was added to cultures immediately after first cleavage. Enzyme was assayed at intervals in homogenates by following the disappearance of added substrate spectrophotometrically. Specific activity was referred to protein. The level of adenosine used had slightly adverse effects, resulting in the formation of plutei with short, blunted arms. The control animals, raised in sea water alone, evidenced a gradual increase in enzyme activity as development proceeded. After 20 hours in adenosine, the specific activity of the enzyme in the experimental embryos was 10–20% less than that in the controls. This was interpreted as due to the adverse effect of adenosine, as noted above. Thereafter a rise in the level of enzyme in the experimental embryos occurred. By 90–96 hours after addition of adenosine, the specific activity in the experimental embryos was from 32% to 85% greater than that in the controls. Induction was also demonstrated by measurements made forty hours after the addition of adenosine to one-day plutei. The adverse effect of adenosine can be mitigated by the use of lower inducer concentrations. Preliminary evidence indicates that this treatment results in a more pronounced elevation of activity than was obtained with higher inducer levels.

Permeability studies on Arbacia punctulata eggs. R. G. FAUST AND A. K. PARPART.

Volume changes of Arbacia eggs were produced by the addition of ethylene glycol or NaCl to sea water. These changes were recorded by means of a photoelectric densimeter. It has been demonstrated by many other investigators that fertilization increases the permeability of the egg membrane. However, the role played by the hyaline layer upon permeability of fertilized eggs has been controversial.

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[1] Pre-doctoral fellow of the National Science Foundation, 1957–58.

Trypsinized fertilized eggs, which have a distinct hyaline layer but no fertilization membrane, were washed for two minutes with a solution consisting of equal volumes of 1 *M* glucose and 0.5 *M* NaCl. Thus, these fertilized eggs had neither a fertilization membrane nor hyaline layer. They were returned to sea water and tested in the densimeter. The permeability of these eggs to water and ethylene glycol was the same as that of normal fertilized eggs having both fertilization membranes and hyaline layers. The increase in permeability caused by fertilization is therefore independent of the hyaline layer or the fertilization membrane.

Direct evidence for a distal retinal pigment dark-adapting hormone in Palaemonetes vulgaris.[1] MILTON FINGERMAN, MILDRED E. LOWE AND BANGALORE I. SUNDARARAJ.

Heretofore, there has been no direct demonstration of dark-adapting hormone for the distal retinal pigment of any crustacean kept under constant illumination. Previous evidence for a dark-adapting hormone was based on differences in rates of dark adaptation between control prawns and those injected with extracts of various organs, followed by transfer to darkness. In the present investigation one-eyed prawns were kept on a black background under a constant illumination of 27 ft. c. The state of the distal retinal pigment was slightly less than midway between the fully light-adapted and the fully dark-adapted conditions; so the presence in extracts of a light-adapting or a dark-adapting hormone could be demonstrated. Boiled extracts of whole eyestalks produced maximal light adaptation followed in two hours by dark adaptation that lasted about five hours. The light-adapting and the dark-adapting effects of extracts of the sinus gland equalled those of the optic ganglia. Extracts of tritocerebral commissures produced slight light and dark adaptation. The supraesophageal ganglia and circumesophageal connectives without the commissures had no dark-adapting effect. With the commissure attached, the light-adapting ability of these tissues was decreased, presumably due to the presence of the dark-adapting hormone in the commissure. Addition of the commissure to extracts of eyestalks similarly decreased their light-adapting potency.

Influence of long-term background adaptation on the lability of chromatophores and the sources of chromatophorotropins in Palaemonetes vulgaris.[1] MILTON FINGERMAN, MURIEL I. SANDEEN AND MILDRED E. LOWE.

To study the effects of long-term background adaptation on endocrine sources and on the target organs, groups of *Palaemonetes* were placed on black and on white backgrounds. At intervals of 2 hours, 1, 2, 4, 6, 8 and 14 days the rate of migration of the dark red pigment was determined after moving the animals to the opposite background. The rates of pigment migration gradually decreased. The midpoint of pigment dispersion, chromatophore stage 3, was reached in 15 minutes by 1-day white-adapted animals and not for 60 minutes by 14-day white-adapted animals. Pigment concentration was less affected. Chromatophore stage 2.5 was achieved in 15 minutes by 1-day black-adapted animals and in 30 minutes by 8-day black-adapted animals. The nervous system source of chromatophorotropins studied was the circumesophageal connectives with the tritocerebral commissure attached. Extracts of these organs from animals kept on black or on white backgrounds for 2 hours and for 14 days were assayed on one-eyed animals for dark red pigment concentrating and dispersing activity. After 14 days of background adaptation extracts from the animals on white had much more dispersing effect, activity of 5.7, than those on black, activity of 0.7, and less concentrating effect, activity of 0.9 compared to 2.9. In animals adapted for only 2 hours the dispersing activity of tissues from animals on white was 2.6, compared to 2.3 from animals on black. The concentrating activity of tissues from animals on white was 1.7 and from animals on black, 2.6. These results offer evidence in support of the hypothesis that these tissues produce hormones, particularly red dispersing substance, which function in normal background responses.

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[1] This investigation was supported by Grant No. B-838 from the National Institutes of Health.

Further studies on the chromatophorotropins of Palaemonetes vulgaris.[1] MILTON FINGERMAN, BANGALORE I. SUNDARARAJ AND MURIEL I. SANDEEN.

Two studies were initiated to define the nature of the antagonistic chromatophorotropins controlling dispersion and concentration of the dark red pigment in *Palaemonetes.* Filter paper electrophoresis of tissue extracts was performed at 5–7° C. for two hours with $M/10$ borate buffer, pH 7.2 at 500 V. and 0.1–0.2 mA. The eyestalks contained an electronegative red pigment dispersing and an electropositive concentrating substance. The supraesophageal ganglia plus the circumesophageal connectives contained an electropositive and an electronegative red pigment dispersing substance. Comparison of extracts of eyestalks, fresh and boiled, revealed that the former produced considerable red pigment concentration and no significant red pigment dispersion while the latter produced much less pigment concentration and a significant amount of pigment dispersion. To analyze this difference sinus glands were separated from optic ganglia and fresh and boiled extracts of each were assayed. Neither fresh nor boiled extracts of the sinus glands produced any pigment dispersion although boiling decreased the amount of pigment concentration produced. Fresh extracts of optic ganglia produced pigment concentration and dispersion. Boiled extracts produced an increased amount of pigment dispersion. Boiling extracts of other parts of the central nervous system likewise increased their pigment dispersing potency. Keeping fresh extracts of eyestalks or other parts of the nervous system at room temperature for four hours was about equivalent to boiling for one minute, the pigment concentrating activity decreased and the dispersing activity increased.

Sodium and potassium exchanges in photosensitized fish red cells. JAMES W. GREEN AND THOMAS A. BORGESE.

The object of the present study has been to examine the action of the fluorescent dye rose bengal on Na and K exchanges in fish red cells. Blood was generally drawn by heart puncture, placed in isotonic NaCl solution and defibrinated. After centrifugation the cells were washed twice in saline and refrigerated until used, generally within two days of collection. Erythrocytes from the elasmobranch *Dasyastis* (sting ray) and the teleosts *Scomber* (mackerel) and *Lophius* (goose fish) were used. Hemolysis curves of cells photosensitized to 2×10^{-6} M rose bengal by exposure to light from a 12 W fluorescent bulb were determined. The times to 50% hemolysis following a two-minute irradiation were: sting ray 41 minutes, goose fish 30 minutes, and mackerel 16 minutes.

For the ion exchange experiments, irradiation times of one or two minutes, of 1.8% erythrocyte suspensions, were used. The suspensions were reconstituted and incubated at room temperature in an agitation roller with either Na^{24} or K^{42} and glucose saline for periods up to 8 hours. All erythrocytes exhibited an increase in Na^{24} and a decrease in K^{42} when photosensitized. The most satisfactory experiments, those with mackerel cells, showed that K^{42} exchanged, in photosensitized cells, only 47.8% as rapidly as the controls while Na^{24} exchanged 200% more rapidly. In the photosensitized cells glucose disappearance was 50% greater than the controls. It is tentatively concluded that the nucleated fish erythrocyte is more sensitive than the mammalian red cell.

A crescent-shaped figure in the hyaline layer of the Arbacia egg. ETHEL BROWNE HARVEY.

A crescent-shaped figure often appears at the surface of the *Arbacia punctulata* egg if the eggs in the "streak" stage, 13 to 30 minutes after fertilization at 23° C., are placed for 5 minutes or longer in N/200 NaOH (in distilled water); this has been found to be the

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[1] This investigation supported by the National Institutes of Health.

most favorable solution. The crescent is darker than the surrounding protoplasm and is sharply outlined in black. It is a curved band with pointed ends, varying in width from about 2 μ to about 20 μ; it is sometimes short but may extend completely around the cell, or nearly so; the egg swollen in this hypotonic solution measures about 130 μ in diameter, the normal egg measures 74 μ. The band tends to be thin in earlier stages. In stages before the crescent is formed, this is represented by a somewhat spherical mass of dark-staining cytoplasm. Thirty-five minutes or more after fertilization, eggs similarly treated do not show a crescent. The crescent occurs in eggs from which the fertilization membrane has been removed, but not in eggs from which the hyaline layer has been removed by calcium-free sea water, indicating that it lies in this layer; it does not extend into the interior of the egg. It does not appear with distilled water alone, without the alkali. Alkalis other than sodium can likewise be used: KOH, $Ca(OH)_2$, NH_4OH.

The crescent is formed in centrifuged eggs, bearing no relation to the stratification; it is also found in the white half-eggs, rather pale but outlined in black; and also in the red half-eggs, small and quite dark. It occurs in parthenogenetic as well as in fertilized eggs.

Since the crescent occurs during the "streak" or "pre-spindle" stage, that is, after the monaster and before the amphiaster, it seems probable that it is connected with the division of the centrosome, two parts of which come to lie one at each pole of the spindle. However, the position and orientation of the crescent do not always seem to correspond with that of the preceding streak.

There is also, with the same treatment with N/200 NaOH, a darkening of the cleavage furrow in dividing eggs, where the hyaline layer becomes thickened, and later in the layer between the two blastomeres. There is no darkening of the micromeres.

Many stains and reagents have been tried in order to determine the chemical nature of the crescent, but nothing has been found to stain the crescent differentially except that there is a slight greenish tinge with methyl green, a bluish tinge with methylene blue, and it stains pinkish lavender with toluidin as does also the hyaline layer. Among other substances tried were: Sudan III, Millon's, Schiff's and ninhydrin reagents, Feulgen stain, Janus green, pyronine, neutral red, aceto-carmine, picro-carmine. No dyes were found to stain a figure in the cytoplasm such as a crescent without the treatment with dilute alkali. Nothing more is seen with a phase microscope than with a light microscope.

Studies on membrane elevation in the eggs of Chaetopterus and Nereis.[1] CATHERINE HENLEY.

It has been suggested by Costello (1958, *Physiol. Zoöl.*) that the waves of contraction which are apparent in the vitelline membrane of the Chaetopterus egg, beginning about 20 minutes after insemination, may be due to the rhythmic release of colloidal material. To test this hypothesis, the effects have been studied of several agents on membrane elevation in the inseminated eggs of Chaetopterus and Nereis.

When fertilized Chaetopterus eggs were cold-treated in pre-chilled filtered aerated sea water (1 to 3° C.), beginning 5 minutes after insemination, for periods of 1½ to 3 hours, there was an asymmetrical exaggeration of membrane elevation. This was first apparent within 10 minutes following the end of cold-treatment, as a localized surface crenulation of the membrane. Gradually, the perivitelline space increased in width, the space being widest at one sector but also discernibly wider than normal for the entire circumference of the egg-membrane complex. In eggs which were treated for longer periods (2½ to 3 hours), the membrane elevation eventually terminated in a bursting of the membrane, so that the eggs were denuded.

Cold-treated Chaetopterus eggs with exaggerated membrane elevation were placed in a solution of gum arabic in sea water. The membranes promptly collapsed back against the surfaces of the eggs, indicating that the colloidal osmotic pressure within the membrane (presumably the cause of membrane elevation) could be "neutralized" by externally applied colloidal osmotic pressure.

Fertilized *Nereis limbata* eggs cold-treated for 90 minutes, beginning 5 minutes after insemination, showed no evidence of exaggerated membrane elevation.

[1] Aided by a grant to Dr. D. P. Costello from the National Institutes of Health, RG-5328.

Uninseminated Chaetopterus eggs which were x-irradiated with doses of 20,000 and 40,000 r and then fertilized showed no exaggerated membrane elevation during the first hour after treatment, in contrast to the results reported by Redfield and Bright (1921) for irradiated Nereis eggs.

Mercaptoethanol and Tetrahymena. George G. Holz, Jr.

The effects of 2-mercaptoethanol on morphogenesis, nuclear division and cleavage have been tested on *Tetrahymena pyriformis* (MTI., V1) whose division was synchronized by temperature changes (5 alternate half-hour periods at 43° and 35° C.). Sixty to 80% were blocked at 43° in the anarchic field stage of stomatogenesis and the anaphase stage of micronuclear division. Normally the first synchronous division occurred 55 minutes after the last 43° exposure, and during the final 10 minutes stomatogenesis and nuclear division resumed and cytoplasmic cleavage began. Mercaptoethanol (0.0004–0.005 *M*) introduced immediately after the last 43° period delayed division. Higher concentrations prevented it; the ciliates remained at the stage characteristic of "heat block."

Application of the thiol (0.025 *M*) during early cleavage retarded but did not prevent fission. Macronuclear division did not occur, micronuclear division was normal, and one daughter was amacronucleate.

Introduction of mercaptoethanol at intervals after the first division showed that the second synchronous division could be delayed or prevented, depending upon the concentration used. When it was added (0.025 *M*) 10–20 minutes before cleavage, the ciliates were blocked at stages of morphogenesis and micronuclear division characteristic of the time of addition. Ciliates treated earlier never began stomatogenesis or micronuclear division. There was no accumulation of a characteristic blocked stage like that produced by cyclic heat treatments.

Starved ciliates introduced to nutrient medium containing mercaptoethanol (0.0025–0.025 *M*) failed to reproduce in 6 hours and grew only to the extent shown by controls. They showed a nuclear constitution and infra-ciliature characteristic of the period between divisions.

The above results demonstrate the lack of a sharp specificity of action of mercaptoethanol on a particular stage of morphogenesis or micronuclear division, and a possible inhibitory action on cell syntheses necessary for growth.

The action of pentahalophenols on oxygen consumption and cell division and on the glucose-6-phosphate dehydrogenase of the eggs of Arbacia punctulata. A. K. Keltch, H. H. Hiatt, C. P. Walters and G. H. A. Clowes.

In extension of previous work, the compounds pentachlorophenol and pentabromophenol were tested at graded concentrations for their possible influence on oxygen consumption and cell division of fertilized *Arbacia* eggs and on the glucose-6-phosphate dehydrogenase activity of extracts of unfertilized eggs. The influence on cell division and oxygen consumption was similar to that previously observed with a large series of nitro- and halophenols. Oxygen consumption was stimulated by both pentahalophenols. As the concentration of phenol was increased, the stimulation increased in degree, reached a maximal value, and decreased. Cell division, on the other hand, was reversibly inhibited. At a concentration of 10^{-6} *M* pentachloro- or pentabromophenol, cell division was inhibited 50 per cent and oxygen consumption was stimulated about 80 per cent. Glucose-6-phosphate dehydrogenase activity was inhibited by these compounds. Fifty per cent inhibition was obtained with 9×10^{-6} *M* pentachlorophenol and 10^{-5} *M* pentabromophenol.

Dehydrogenase activity in developmental stages of Spisula as measured with a tetrazolium salt. Evelyn Kivy-Rosenberg, Karen Steel Kagey and Joseph Cascarano.

A quantitative estimate of endogenous dehydrogenase activity during developmental stages in Asterias had been sought earlier (Kivy-Rosenberg and Zweifach, 1956, *Biol. Bull.*, **111**) using two tetrazolium salts as indicators—TTC and NT—but the toxicity of these salts made such studies not feasible. Since then investigations on specific substrate-dependent dehydrogenase activity have been in progress.

A comparative study was undertaken of Spisula but endogenous activity again was not feasible since INT, which is a more active acceptor than TTC or NT, is highly insoluble in sea water. Attention was turned once again to studies of specific substrate-dependent dehydrogenase activity, using several stages in development including uninseminated and inseminated eggs. Samples of both fresh and frozen eggs were incubated at 37–38° C. for one hour in a medium containing one of a series of substrates including succinate, and with DPN as cofactor, alpha-glycerophosphate, glucose, glutamate, malate, lactate, beta-hydroxybutyrate, ethanol. The formazan was extracted and amounts of reduced tetrazolium determined photometrically. This activity was expressed as micrograms of formazan per milligram of dried tissue.

As in Asterias, malate-dependent dehydrogenase activity was the greatest and alpha-glycerophosphate dehydrogenase activity less but still second. The other dehydrogenases all showed some, though limited activity. Quantitatively, Spisula shows more uniformity than Asterias. With respect to metabolic changes during development, limited observations indicate that both malate and aplha-glycerophosphate dehydrogenases become less active following insemination.

In Spisula the quantity of eggs is quite limited and so microchemical determinations were made to parallel macrochemical ones. Homogenates of both fresh and frozen uninseminated and inseminated eggs, first cleavage, and early larvae were incubated in a similar manner. The substrate-dependent dehydrogenase activity was expressed as micrograms of formazan per milligram of protein. With this method, the malate and alpha-glycerophosphate-dependent dehydrogenase activity ranked as it had in the whole egg. There is, however, a discrepancy seen between reactions of the organized cell and disorganized material of the cell which will be further investigated. In the homogenized state there is a rise in malate-dependent dehydrogenase activity following insemination, rather than a fall. This is, in general, true of alpha-glycerophosphate dependent activity also.

Changes in the levels of triphosphopyridine nucleotide in the eggs of Arbacia punctulata subsequent to fertilization: Presence of pyridine nucleotide transhydrogenase and diphosphopyridine nucleotide kinase. STEPHEN M. KRANE AND ROBERT K. CRANE.

Glucose utilization in the eggs of *Arbacia punctulata* is predominantly via pathways requiring triphosphopyridine nucleotide. The amount of glucose metabolized by these pathways has been shown to increase strikingly in the 24-hour embryos compared to the unfertilized eggs. To determine some of the factors controlling the rate of carbohydrate utilization, therefore, the steady-state concentrations of oxidized (TPN) and reduced (TPNH) triphosphopyridine nucleotide were measured fluorometrically. TPN was assayed on neutralized trichloracetic acid extracts using TPN-specific isocitric dehydrogenase; TPNH on neutralized sodium carbonate extracts using TPNH-specific glutathione reductase. Recovery of added TPN was 100 per cent. Recovery of added TPNH varied from 33 to 56 per cent and the use of an internal standard was required. TPN levels in unfertilized eggs and 24-hour embryos were 11 and 14 millimicromoles per gram wet weight, respectively, values which are close to the lower limit of sensitivity of the method used. In contrast, TPNH levels in four experiments on unfertilized eggs averaged 19 millimicromoles per gram (range 7–32) and in 24-hour embryos 67 millimicromoles per gram (range 57–90). Eggs one hour after fertilization (two assays) contained 57 and 61 millimicromoles of TPNH per gram. Since the concentrations of TPN were low compared to TPNH, pyridine nucleotide transhydrogenase activity was assayed fluorometrically using the 3-acetylpyridine analog of DPN as electron acceptor. In homogenates at 27° C. rates as high as 14 micromoles per hour of TPNH converted to TPN per gram of eggs were found. Activity of DPN kinase was also found but the presence of inhibitors in the crude homogenate prevented the determination of absolute rates for this enzyme. Further work will be needed to establish unequivocally the very striking increase in TPNH level and the apparent variation in transhydrogenase activity incident to fertilization and development of the eggs. Similar assays on Arbacia sperm are projected.

The biological and chemical mechanisms of protein utilization by Hydra. HOWARD M. LENHOFF.

By inducing a feeding response in Hydra with reduced glutathione it was possible to make them ingest tissue containing protein labeled with radioactive sulfur. The fate of the ingested protein was studied by chemical fractionation and radioautography. The results demonstrated that: (1) The protein was not digested in the gastrovascular cavity, but rather the tissue was broken down into small particles; no significant hydrolysis of the protein to polypeptides or amino acids occurred extracellularly. (2) About 80–90 per cent of the food particles were phagocytized by the gastrodermal cells within 5 hours after ingestion of the food into the gastrovascular cavity. (3) only the gastrodermal cells in the upper two-thirds of the body tube were active in engulfing most of the food; a very slight amount of food particle engulfment occurred in the lower parts of the tentacles. (4) The initial products of intracellular protein digestion were alcohol-soluble proteins or polypeptides. The presence and function of alcohol-soluble proteins in animal tissues has hitherto never been described. Kinetic experiments demonstrated that these unusual proteins supplied precursor material for the synthesis of alcohol-insoluble proteins. Thus, the alcohol-soluble proteins formed by Hydra seem to act in a similar capacity as do the alcohol-soluble storage proteins of plants, the prolamines. (5) During asexual reproduction about 15–25 per cent of the parent's radioactivity was distributed to each bud, depending upon the conditions of growth. (6) Twenty-five per cent of the radioactivity was excreted into the environment during five days of starvation. (7) The Hydra egested its solid wastes by inflating itself with water after most of the food particles were engulfed. When this water was released, most of the solid wastes were flushed out.

Experimental induction of cleavage furrows in the Arbacia egg.[1] DOUGLAS MARSLAND AND WALTER AUCLAIR.

As previously reported, premature furrows, which always cleave the egg at right angles to the centrifugal axis, can be induced at various times up to 35 minutes ahead of the normal cleavage schedule. The induced furrows appear in a high percentage of the eggs, starting about 3 minutes following a 4-minute period of centrifugation at high pressure (8–10,000 lbs./in.^{2}) and high force ($41{,}000 \times g$).

The treatment always ruptures the nuclear membranes, liberating Feulgen-positive material which comes to lie in or near the mitochondrial zone. Less drastic treatments may break the nuclei, however, without inducing the reaction. In fact, the furrowing reaction has only been observed when certain cytoplasmic vacuoles are also broken. These vacuoles are somewhat smaller than the echinochrome vesicles and each vacuole contains from 1–4 granules which stain metachromatically (red) with toluidine blue. Eggs receiving a non-inducing centrifugation show hundreds of these vacuoles in the hyaline and mitochondrial zones, but after an inducing treatment, the eggs show only a diffuse non-granular metachromatic coloring in the hyaline zone, especially near the oil cap and in the neighboring perivitelline space. Numerous metachromatic vacuoles are also observed in close association with the peripheral parts of the asters in non-induced centrifuged eggs; and during telophase some of these vacuoles are seen to move peripherally into the cell cortex.

The data suggest that the metachromatic granulated vacuoles play some role in the induction of the furrowing reaction. It is suggested that induction may depend upon the liberation of material from the vacuoles after they have reached the cell cortex. However, material from the nucleus may also be involved, since furrow-inducing treatments always rupture the nuclear as well as the vacuolar membranes.

Graded and all-or-none electrical activity in insect muscle fibers. F. V. McCANN, R. WERMAN AND H. GRUNDFEST.

In muscle fibers of *Romalea microptera* modifications were produced in the electrically excitable responses by applying divalent alkali earth cations. The effects were analyzed with intracellular stimulations and recording. Confirming earlier work, normal electrically excitable responses of the muscle fibers, whether evoked by neural stimuli or by intracellular depolarizations, are graded. They arise with vanishingly brief latency, are small and decrementally propagated. Maximal directly evoked electrical responses produce only local visually observable contractions. When 10% of the external Na^+ is replaced with Ba^{++}, spontaneous repetitive firing occurs in the fast axon and in the muscle fibers. Hyperpolarization of the latter stops their repetitive discharge. Frequently, however, no postsynaptic potentials are then disclosed. Brief direct stimuli also induce repetitive firing, without neural activity. Therefore, Ba^{++} can initiate repetitive activity in the muscle fibers by direct action. Membrane conductance is usually decreased in the presence of Ba^{++}, the time constant and space constant both increasing while the resting potential is unchanged. The threshold for electrical stimulation decreases markedly. Probably as a result of these several factors, the electrically excitable activity changes from graded to all-or-none responsiveness. Simultaneous recording with several microelectrodes shows that this change occurs only in some regions of the fiber. Spikes therefore may arise at a site some distance from an intracellular stimulating electrode, and sometimes at several sites independently, then propagating into other regions that are gradedly responsive. The duration of the response is greatly increased. Higher concentrations of Ba^{++} further increase spike height and markedly prolong the responses which then resemble spikes of cardiac muscle and may last up to 30 seconds. Brief repolarization by an inward current pulse abolishes the spike. The current required to do this depends on the membrane potential when the pulse is applied. The threshold is lowered and the response amplitude and duration are increased by high concentrations of Ca^{++}, but the magnitudes of these changes are relatively small with this ion. On the other hand, Sr^{++} appears to be almost as effective as is Ba^{++} in converting the gradedly responsive, electrically excitable membrane into the regenerative variety which produces all-or-none spikes.

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[1] Work supported by grant C-807 from the National Cancer Institute, U.S.P.H.S.

Hyperglycemia and islet damage after intracardiac injection of alloxan in toadfish. P. F. NACE, J. E. SCHUH, L. R. MURRELL AND A. D. DINGLE.

Histological and blood sugar studies of toadfish injected with alloxan by gill arch, subcutaneous, intraperitoneal and intramuscular routes have shown very variable responses to doses of 400 to 800 mg./kg. A series of approximately 200 experimental and 200 control fish has shown a very consistent response to the intracardiac injection of 10% iced aqueous alloxan. For an unnumbered lot of Eastman Kodak alloxan, a dose of 700 mg./kg. was effective in almost all cases, with very low mortality. Other lots tested have required higher or lower dose levels.

Marked hyperglycemia was observed three hours after treatment. This persisted for about two days. From 2 to 6 days after treatment, a gradual decline to normal levels was found. Principal islets of animals sacrificed in the first two days after treatment exhibited extensive beta cell damage, with nuclear pycnosis and cytoplasmic degeneration. Islets fixed 6 days after treatment appear nearly normal, with small necrotic foci.

The course of blood sugar and cellular changes in this animal, *Opsanus,* differs markedly from that seen in the catfish, *Ictalurus,* in which both blood sugar elevation and cell damage persist without apparent trend toward recovery.

This work was supported by Research Grant A-1129, National Institute of Arthritis and Metabolic Diseases, U.S.P.H.S.

The effect of bacterial endotoxins and biogenic amines on the phagocytic behavior of endothelial elements in the frog, Rana pipiens. ARNOLD L. NAGLER AND BENJAMIN W. ZWEIFACH.

Although bacterial endotoxins are not lethal in the frog (*Rana pipiens*) in any dose, extracts of *E. coli* (500 μg/40 gm.) administered intravenously facilitated the clearance (as measured photometrically) of a carbon-gelatin suspension from the bloodstream; values for K (phagocytic index) were 0.132 at 45 minutes (26 animals), in contrast to control values of 0.045 in 30 animals (a 300% increase). This stimulating action was no longer evident after two hours.

In search for mediators of this effect, frogs were pre-treated with 48/80 and polymyxin B, agents known to release histamine, 5-hydroxytryptamine (serotonin), norepinephrine and heparin. Both drugs counteracted the stimulating action of endotoxins on RES clearance. Various biologic amines were injected i.v.—epinephrine, norepinephrine, serotonin and histamine. Epinephrine was the only agent with a stimulating effect on the RES. As anticipated, an adrenergic blocker, dibenzyline, abolished the endotoxin effect on the RES. Dibenzyline did not counteract the stimulating action of epinephrine, suggesting that this effect was not due to its pressor properties. When equivalent amounts of serotonin were mixed with epinephrine, there was no stimulation of RES function. Passive transfer of blood, removed after the frogs had received polymyxin B or 48/80, indicates the presence of an inhibitory agent, 1.2 ml. of heparinized blood serving to counteract the usual stimulating action of endotoxin and epinephrine on the RES. Histologic inspection reveals that normally the uptake of carbon is restricted to the liver and spleen. Increased phagocytic function, induced by endotoxin or by epinephrine, is associated with extensive deposits of carbon along the peritubular capillaries and glomerular loops. Agents which prevented stimulation of the RES, prevented uptake of carbon by the kidney vessels.

The data suggest that in the frog reno-portal system, endotoxins result in a more rapid clearance of particulate materials from the bloodstream through an effect primarily on vessels of the kidney mediated by the release of epinephrine.

Changes in behavior of the cell wall and cytoplasm due to injuries in Nitella flexilis. W. J. V. Osterhout.

(I) When an uninjured cell of *Nitella flexilis* was placed for a few minutes in 0.01 per cent cresyl blue solution at pH 9 the dye in molecular form penetrated rapidly into the cell and dissociated in the acid sap. When this cell was transferred to a buffer solution at pH 9 containing no dye, little or no dye escaped from the cell. The cell wall remained pale violet, and the vacuole appeared deep blue.

(II) A cell was bent until a protoplasmic mass was formed as a result of the injury. This cell was placed in the dye solution at pH 9 as in (I). The dye penetrated rapidly into the uninjured parts of the cell and collected in the vacuole while the injured spot appeared less colored until the dye became uniformly distributed in the vacuole. When this cell was transferred to a buffer solution at pH 9 as in (I) the dye came out from the injured spot but not elsewhere. The injured spot became more stained while the rest of the cell became less stained.

(III) If an uninjured cell was placed for a few minutes in 0.05 per cent dye at pH 5.5 the cell wall became purple but no dye was found in the vacuole. When this cell was transferred to a buffer solution at pH 9 the dye from the cell wall rapidly penetrated the cell until the cell wall became pale violet and the vacuole deep blue.

(IV) When a cell was bent as in (II) and was placed in the dye solution at pH 5.5 as in (III) the cell wall was deeply stained after a few minutes except at the injured spot which was much less stained. There was no dye in the vacuole. When this cell was transferred to a buffer solution at pH 9 as in (III) the dye rapidly penetrated the uninjured parts of the cell and collected in the vacuole but the injured spot remained much less stained.

These results indicate that the injuries alter the cell wall and the cytoplasm.

Changes in permeability to an acid dye due to protoplasmic lesions in Nitella flexilis. W. J. V. Osterhout.

(I) Single internodal cells of *Nitella flexilis* were employed.

(II) Buffer solution at pH 5.5 was made up in 0.02 M phosphate buffer mixture. Buffer solution at pH 8 was made up in 0.02 M phosphate or borate buffer mixture. The concentration of the dye solution was 0.05 per cent unless otherwise stated. Staining of the protoplasmic mass was determined after two minutes' exposure to the dye solution. The exit of the dye was determined after five minutes in a buffer solution containing no dye unless otherwise stated.

(III) The dye did not readily enter the cells unless they were injured.

(IV) The cell was bent until a protoplasmic mass was formed as a result of the lesion. This was rapidly stained in the dye solution at pH 5.5. The same result was obtained with the dye in distilled water.

(V) If a cell was placed in the dye solution at pH 8 the dye did not stain the protoplasmic mass rapidly. There was very little staining after two minutes' exposure.

(VI) If a cell was immersed for five minutes in the buffer solution at pH 8 and then transferred to the dye solution at pH 5.5 there was a rapid staining of the protoplasmic mass.

(VII) The dye came out from the protoplasmic mass into distilled water, into a buffer solution at pH 8 or at 5.5, all containing no dye and also into the buffer solution at pH 8 containing 0.05 per cent dye.

(VIII) Since the color of the dye at pH 8 became paler after a few minutes higher concentrations of the dye up to 0.5 per cent were used and the same results were obtained.

These results indicate that the permeability to the dye is altered at the point of bending so that the protoplasmic mass can take up an acid dye rapidly when the rest of the cell remains unstained. The staining of the cell wall is too slight to play an important role here.

Inhibitory effect of electrolytes on the penetration of organic molecules into Nitella flexilis. W. J. V. OSTERHOUT.

(I) A basic dye, azure B, penetrated into the cells of *Nitella flexilis* more rapidly at higher pH values in which the dye was largely in molecular form. The dye dissociated in the acid sap and accumulated since it could not come out of the cell rapidly in this form.

(II) The control cells were kept in solution A formerly described by the author. These cells were transferred to 0.005 per cent dye solution at pH 8 (in 0.007 M borate buffer mixture) at 22° Centigrade, and after one minute there was 0.008 per cent dye in the vacuole. This was taken as a standard with which other experiments were compared.

(III) Previous exposure to distilled water for one hour did not alter the rate of penetration of the dye as compared with the standard.

(IV) Previous exposure to 0.001 M $CaCl_2$ solution for five minutes did not alter the rate of penetration of the dye as compared with the standard. The same results were obtained when similar experiments were made with $MgCl_2$, $MgSO_4$, and $LaCl_3$. All pH values were adjusted.

(V) Previous exposure to 0.005 M KCl solution for five minutes brought about 45 per cent decrease in the rate of penetration of the dye as compared with the standard. The same results were obtained when similar experiments were made with KNO_3 and K_2SO_4.

(VI) This inhibitory effect of the salts with monovalent cations was completely abolished when the cells were subsequently exposed for five minutes to 0.001 M salts with bivalent and trivalent cations (under IV).

(VII) The concentration of the halides in the control sap was 0.12 M. This remained unchanged when the cells were exposed to the salt solutions mentioned under (IV) and (V) for one-half hour.

These results confirm those obtained by Marian Irwin in experiments on the inhibitory effects of sodium salts on the penetration of cresyl blue and subsequent abolition by salts of bivalent and trivalent cations.

Rate of recovery of centrifugally-deformed mast cells as a function of age in the rat. JACQUES PADAWER,[1] DOUGLAS MARSLAND[2] AND WALTER AUCLAIR.

Rat peritoneal fluid, containing numerous free-floating mast cells, was centrifuged at 41,000 × g and 12,000 lbs./in.^{2} pressure long enough to deform appreciably most of the cells. By this procedure, pressure-induced cytoplasmic solation allowed the normally spherical mast cells to elongate. Rates of return to spherical shape were then measured both on a population and on an individual cell basis. Two age groups were investigated, 7–9-week and 11–12-month old rats. In the young adult animals, population recovery was much faster than in the older rats. From a time-lapse photographic study of individual cells, this difference was shown to result from an appreciable lag prior to the inception of rounding in many of the cells from the older rats, as well as from morphological readjustments involving transitions from one type of deformation to another, a process not commonly observed with cells from the younger rats. Shortening of deformed mast cells was found to proceed as a definite function of time. A few non-spherical mast cells normally occurred in the peritoneal fluid of the older rats and these, too, eventually rounded up *in vitro*. From the data, it appears that the differences observed between the two age groups studied are ascribable to all the cells of the respective populations.

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[1] Supported by grant from the American Heart Association.

[2] Supported by grant C-807 from National Cancer Institute.

A novel method for correcting astigmatism in electron microscopes. DELBERT E. PHILPOTT.

Most recently produced electron microscopes are capable of resolution of approximately ten Angstrom units. However, this resolution lasts for a very short period of time and then slowly declines. This is due to contamination of the objective aperture which is usually necessary to provide adequate contrast in the final image. Externally compensatable microscopes make it possible to visually correct most of the astigmatism while viewing the defect with some suitable specimen. The final correction must be done on photographic plates. The purpose of the following method is to provide visual correction down to the limit of resolution.

Two sets of coils, one above the other, when placed on opposite sides of an electron beam can be made to wobble the beam when it is out of focus if alternating current is fed to these coils. The disadvantage of the above is that any astigmatism in any direction except ninety degrees to the beam wobbler will cause the focus to be off proportionately to the degree of astigmatism. By placing another set of coils ninety degrees to the first set, and making the unit mechanically rotatable, one set can be put in the direction of astigmatism and the other perpendicular to the beam (where no astigmatism exists). The microscope will now focus properly with one set of coils and overfocus with the second set. External compensation can now be applied until the beam wobbling in the direction of astigmatism ceases. Since the beam wobbling increases the ability to see the astigmatism, visual correction can be carried out without the need to resort to photographs. This saves time and correction carried out during operation is not subject to mechanical or electrical change before the pictures are taken.

Conduction in Phascolosoma fusiform muscle. CHARLES L. RALPH AND C. LADD PROSSER.

The fusiform muscle of *Golfingia* (*Phascolosoma*) *gouldi*, a non-striated retractor, is a thread-like structure along the mesentery of the ascending intestine. Its fibers are approximately 4 μ in diameter, 1 mm. in length and have a helical configuration when contracted. Electrical stimulation of the muscle evoked a twitch-like contraction lasting 8–10 seconds. Frequently, repetitive contractions, occurring at regular intervals and continuing for several minutes, followed a single stimulation. A quick stretch of 1–2 mm. produced a twitch essentially like that evoked by electrical stimulation. These responses were graded in tension and latency according to the amount of stretch applied. Tetracaine, d-tubocurarine, and high calcium concentrations all modified the twitch by slowing the rate of tension development and extending its duration 4–5 times the normal length. Acetylcholine stimulated the muscle, but physostigmine did not potentiate its effects. Although there is a nerve extending the length of the muscle there is no evidence that it mediates excitation. Tetracaine (10^{-4}) failed to block conduction. Degenerated muscle segments showing no nerve fibers when examined with methylene blue still responded to stretch and electrical stimulation. High amplification oscilloscope recordings from external electrodes failed to show nerve spikes preceding the muscle action potential. The latter were propagated at about 1 cm./sec., characteristic of muscle conduction. When a portion of the muscle was prevented from moving, as by wrapping the middle part several times around a glass rod, there was no activity beyond the fixed point. Several methods of suspending the muscle demonstrated that unless the stimulated muscle was allowed to develop tension and produce stretch in a non-excited region, there was no propagation of excitation. It is concluded that in the fusiform muscle conduction is effected by means of intercellular stretch.

A photosensitive pigment from the dorsal skin and eyespots of the starfish, Asterias forbesi.[1] MORRIS ROCKSTEIN, JANICE COHEN AND SANFORD A. HAUSMAN.

Improvement in extraction procedures, consisting chiefly of an increase to five of the number of acid buffer "wash extractions," resulted in the elimination of an overlying violet pigment and a resultant "rosy peach" to the 2% aqueous digitonin extract of the dorsal skin. Despite this visible change in color, the λ max of this pigment remained unchanged at 495 mμ; the minimum absorbance was shifted somewhat to about 385 mμ. However, the lowering of the D_{min}/D_{max} from values of as high as 0.7–0.8 (*cf. Biol. Bull.*, **113**: 353–354, 1957) to as low as 0.45 indicated as improved purity of the extracted photosensitive pigment. The difference spectrum was, as expected, essentially identical with those obtained for exposures to lower wave-lengths (340 to 550 mμ). However, the "reverse bleaching" effects of short-term exposures to longer wave-lengths (600 to 700 mμ), with an identically *reciprocal* difference spectrum, reported earlier, could be reversed by increasing the exposure time at such wave-lengths to 4.5 hours. It is thus apparent that at longer wave-lengths the photosensitive pigment of the starfish is capable of regeneration, provided that such exposure is limited (*cf.* Hubbard, R., and St. George, R. C. C., *J. Gen. Physiol.*, **41**: 501, 1958). Heating in boiling water bath for five minutes shifted the peak absorbance to 455 mμ with a visible color change to a yellow-brown peach hue accompanied by the deposition of a whitish precipitate. A Biuret test of this precipitate was positive. A Biuret test of an unheated digitonin extract of 250 pigment spots was also positive, indicating at least 10 mg. of protein per 250 eyespots. It appears as though the photosensitive pigment in the skin and eyespots of *A. forbesi* does not involve the splitting of a retinene-like compound and a protein in the presence of effective light intensities. Indeed, the splitting off of a protein moiety is accomplished only with extreme denaturation. A Carr-Price test on a chloroform extract of methanol-treated pigment from non-dark-adapted eyespots gave a positive result.

Artificial hybridization between two species of Menidia (silverside fishes). IRA RUBINOFF AND EVELYN SHAW.

M. menidia and *M. beryllina* are sympatric species in the Cape Cod area. They occupy similar ecological niches and are frequently taken together in a seine haul. Moreover, the gonads of both species are ripe at the same time during June and early July, suggesting that these fish may be capable of interbreeding. However, no hybrids have been found among the many specimens collected in the field. To determine whether or not these fish can hybridize, reciprocal crosses were made in the laboratory with 16 adult *M. menidia* and 51 adult *M. beryllina*. In the classification of these species, the main distinguishing character is the number of rays in the anal fin; adult specimens of *M. menidia* have 22–25 rays (mean, 23.3), those of *M. beryllina* have 14–17 rays (mean, 15.2). Since these ranges in the adults do not overlap, this character was analyzed in the F_1 generation.

Good yields of fertilized eggs were obtained from both reciprocal crosses. All groups began hatching 9 days after fertilization, and in most specimens the rays could be counted by the 24th day after hatching. The average numbers of fin rays of both hybrid crosses fall between those of the parents. The mean numbers and ranges of the hybrid and control groups were as follows; *M. menidia* ♀ × *M. beryllina* ♂, 20.8 (19–23); *M. beryllina* ♀ × *M. menidia* ♂, 18.6 (17–20); *M. menidia* ♀ × *M. menidia* ♂, 22.8 (22–25); *M. beryllina* ♀ × *M. beryllina* ♂, 15.8 (14–17).

The fact that these forms can hybridize in the laboratory raises questions about the natural isolating mechanisms that tend to keep them taxonomically discrete.

[1] This research was supported in part by a grant from the National Science Foundation (NSF G-3517).

The effect of x-irradiation of the early fish embryo. ROBERTS RUGH AND ERIKA GRUPP.

The poikilothermic eggs of Fundulus (fish) are radio-resistant when compared with eggs of homoiothermic forms. There is a progressive decrease in the sensitivity with early developmental changes, the pre-cleavage stage being the most sensitive and the post-gastrulation stage the most resistant. The relative values were 500 r to 10,000 r.

This study was designed to determine whether the nervous, muscular, or circulatory systems might be specifically affected following x-irradiation of pre-differentiation stages. It was found that exposure prior to gastrulation, even at the 2–4-cell stage, could affect neural development without comparable effects on the muscular or circulatory systems. Fish embryos were produced without heads, without eyes, and with various degrees of deletion of anterior neural elements. In these same embryos, which survived well but did not hatch, there was normal but slightly retarded pulsation of the heart (sometimes even without corpuscles), and muscular movements of the caudal end. Thus it appears that failure of development of the nervous system may be caused some time before the onset of neural differentiation while the circulatory and muscular systems are not comparably affected. The effect appears to be largely cephalic since there is response of caudal structures to tactile stimulation.

Ionic regulation in a spider crab. RICHARD C. SANBORN.

Animals respond to ionic and osmotic changes in their environment by *regulating* the ionic and osmotic concentration of their body fluid or by allowing the body fluid to reflect external changes and *adjusting* the tissues to such variations. Examination of several organs of an animal the osmotic concentration of whose blood varies with changing external concentration, *Libinia emarginata,* shows that they are surrounded by a tough, multicellular connective tissue sheath. I have tested the ability of the sheath surrounding the supraeoesophageal and thoracic ganglia of this animal to regulate the osmotic and ionic composition of the tissues within. Isolated ganglia with the cut ends of the nerves ligatured were treated with varied concentrations of some twenty compounds; changes in weight and in internal concentration of relevant compounds and ions were measured by appropriate techniques. The tissues, when surrounded by an intact sheath, undergo net gains or losses of water, glycerol, and the following ions: bicarbonate, chloride, magnesium, potassium, sodium and probably calcium. In parallel experiments no net gain or loss of glucose, sucrose, sulfate, or lithium is observed at external concentrations varying from 100 to 1600 milliosmoles per liter. When the integrity of the sheath is interrupted, the final concentration and the rate of penetration of the ions and compounds are markedly changed.

Preliminary measurements with a micro-electrode indicate that the potential between sea water and the extracellular space inside the sheath is about 30 mv., while the neurons within the sheath have resting potentials of 60 to 80 mv. When soaked in sea water, the ensheathed ganglia lose potassium and take up sodium and chloride. Similar behavior was described for leg muscle fibers of this species by Stephenson (1955, *Biol. Bull.*). On the basis of these experiments it seems that the adjustment of *Libinia* to environmental changes in salt concentration is carried out, in large measure, by the connective tissue sheath rather than by the cells or by osmoregulatory organs. Similar mechanisms have been shown to operate in another arthropod group, the insects.

Aided by a grant from the National Science Foundation.

Water relations of the Spisula egg. VICTOR SCHECHTER.

The phase of the work here reported has been directed chiefly toward the effect of water, introduced under hypotonic conditions in the medium, upon nuclear dynamics.

1. Cleavage furrows continue to appear after nuclear activity has been inhibited by means of water narcosis. It is a significant implication that the cortical phenomena of cleavage are not rigidly locked to the mitotic figure but are, to some extent at least, autonomous.

2. The germinal vesicle is less sensitive to water than are the pronuclei. The critical point for inhibition of maturation does not occur until a dilution containing only 40% sea water, while male and female pronuclei cease their interaction in 60% sea water.

3. There is a slight but definite difference in the relative sensitivity of the micromere and the macromere of the first cleavage, resulting in a transient 3-celled stage, as one blastomere precedes the other in cleavage.

4. Recovery time of the egg as a whole from water narcosis is of lesser or of equal duration to exposure time down to a dilution of 50% sea water. Beyond this point, recovery time exceeds exposure time, thus defining the limits of injury to the protoplasmic complex.

5. There is proof that the plasma membrane at the surface of the egg, like the nuclear membrane itself, is impermeable to the osmotic constituents of the nucleus.

Urethan inhibition of cleavage in the Chaetopterus egg and its antagonism by various substances. HERBERT SCHUEL.

When eggs of the marine worm *Chaetopterus pergamentaceus* are placed in a 1% solution of urethan (ethyl carbamate) in sea water 5 minutes after insemination, cleavage is inhibited and the characteristic increase in cytoplasmic viscosity, the "mitotic gelation," does not take place. Ordinarily when eggs are allowed to remain in 1% urethan (at 21° C.) very few if any eggs begin to divide until about 4 hours after insemination. The percentage of eggs found to have divided under these conditions during this period ranges from 0% to 6%. The addition of small amounts of proteolytic enzymes, of isotonic calcium chloride, or of certain basic substances makes it possible for more of the eggs to cleave while still in the urethan solution. Thus at two hours after insemination, from 10% to 30% of such eggs have already divided. The following substances were found to be effective: 0.1% to 0.0001% trypsin, 0.1% to 0.0001% chymotrypsin, 0.037 *M* to 0.007 *M* calcium chloride, 0.01% to 0.00001% histamine, 0.01% to 0.0001% protamine, 1.0% to 0.0001% arginine, and 1.0% to 0.0001% lysine. In no case, however, is there more than a temporary recovery, because the eggs are able to divide only a few times and do not develop into larvae.

Additional evidence for somatic reduction in the metamorphosis of the ileum of mosquitoes by the use of tritiated thymidine.[1] JOSEPH E. SCHUH, S. J. AND GEORGE CARANASOS.

During the larval life of the mosquito, the epithelium of the ileum grows by increase in size of cell rather than by cell multiplication. Berger (1938) described the origin and fate of these large multiple complexes containing 96, 48, 24, 12 and the diploid number of 6 chromosomes. The larval epithelium does not undergo histolysis but these large cells undergo several somatic reduction divisions during metamorphosis, giving rise to the numerous small cells of the adult hind gut. Tritiated thymidine gave promise of being a very suitable tool for a further study of this unique phenomenon.

Larvae of *Aedes aegypti* were exposed to varying amounts of tritiated thymidine in their culture medium in various stages of larval development and for different lengths of time. Newly hatched larvae, late first instar, second, and third and fourth instar larvae were treated for 12, 24 and 48 hours with 25, 50 and 100 μC of tritium per 10 ml. of culture medium. Pupal hindguts were dissected in insect Ringer solution and aceto-orcein smears prepared. Cover-slips were removed by the dry ice method and radioautographs prepared by placing Kodak Ltd. AR 10 Radioautographic Stripping Film over the specimen. The film was exposed for from 5 to 10 days and developed according to the method of Taylor (1956).

Preliminary results indicate that the later the treatment is begun the fewer "hot" nuclei are found in the metamorphosing and metamorphosed hind gut. The hind guts of pupae developed from larvae treated in the third and fourth instar for 12 and 24 hours contained both "hot" and "cold" multiple complexes, side by side. These were in the late prophase stage and were coming up for their first division. The hind guts of pupae from larvae treated shortly after hatching and exposed to the tritium until pupation or late fourth instar showed the majority of the cells of the metamorphosed hind gut to be "hot."

Affinity of tissues in reconstituting tunicates. SISTER FLORENCE MARIE SCOTT.

Tunicates provide a rich source of material for the study of tissue affinity. The organs are simple, consisting of a single layer of cells and basement membrane; histological charac-

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[1] Supported by a grant from the U. S. Atomic Energy Commission.

teristics are easily discernible; tissues are versatile in their capacity to regenerate and reconstitute. The entire animal, moreover, can be used in a variety of experiments constructed to investigate the behavior of whole, though simple, systems in their ability to recognize their related structures and to re-establish former associations. Zooids of *Amaroecium constellatum,* twelve hours after attachment, when adult organization has differentiated but feeding activities have not yet commenced, are divided into three parts to insure separation of organs and are immediately dissociated into floating masses of tissue within the tunic. The experimental animals are implanted in tunics of older evacuated individuals which provide attachment and facilitate their return to running sea water for optimal conditions of development. The tissues begin promptly to reconstitute themselves. They retain their histological characteristics throughout the period of recovery from maceration. Necrotic cells are extruded into the host tunic space. Parts of organs aggregate together and recover their former organic status. After twelve or twenty-four hours of recovery, the parts of systems re-assemble into their original relationships. They accomplish this reunion by a process of directed growth, cells from the anterior end of the oesophagus growing to meet corresponding masses of cells from the oesophageal funnel at the base of the branchial basket. If there are masses of caudal elements or cellular detritus in their paths, they move around the intervening masses to effect the union. In cases where fragments of the organs are too widely separated to become re-integrated into their original positions in a system, the fragments reconstitute themselves and regenerate anterior and posterior sections and, then, attach themselves to their parent structure in the proper linear axis. There may be, therefore, a smaller pharynx attached to the main pharynx or a hernia-like segment attached to the intestine.

Inhibiting action of a triphenylethanol derivative on the development of eggs of Arbacia punctulata and on the fertilizing capacity of the sperm.[1] SHELDON J. SEGAL AND ALBERT TYLER.

The anti-fertility action in rats of 1-(*p*-2-diethylaminoethoxy-phenyl)-1-phenyl-2-*p*-anisylethanol (referred to as MER-25) has been attributed to interference with pre-implantation zygotic development. It has been proposed that MER-25 may have a direct detrimental effect on the zygote or may influence it indirectly, by altering the oviducal environment. Tests of possible direct action were carried out on the gametes and developing zygotes of the sea urchin since this avoids the complicating conditions of internal fertilization and viviparity. A concentration of $2 \times 10^{-5}\%$ of MER-25, added to the sea water medium 5 minutes after fertilization, blocks first cleavage of over 50% of treated eggs. Those which initiate development do not progress beyond the blastula stage. Higher concentrations of the compound ($2 \times 10^{-4}\%$) prevent the normal onset of development in virtually all the newly-fertilized eggs. At lower doses ($2 \times 10^{-7}\%$) the compound's inhibitory activity is also manifest, although a small percentage of the zygotes (⅕ the expected number) may progress through gastrulation and develop into normal plutei. The blocking effect of MER-25 on fertilized eggs may be reversed by washing after exposure to even highly active concentrations. When unfertilized eggs are treated with the above-mentioned concentrations of MER-25 a normal number remain fertilizable but developmental arrest, as noted previously, occurs. Washing unfertilized eggs removes the inhibiting effect. The effectiveness of MER-25 is not enhanced significantly by prior trypsin treatment of the unfertilized eggs. The compound also inhibits both motility and fertilizing capacity of spermatozoa. Ninety to ninety-nine per cent of the spermatozoa in a 1% sperm suspension become immotile within 3 minutes in a $2 \times 10^{-4}\%$ solution; the suspension is unable to fertilize normal eggs.

Structure-activity-relationships concerning the inhibitory activity of synthetic estrogens and some triphenylethanol derivatives on developing eggs of Arbacia punctulata.[1] SHELDON J. SEGAL AND ALBERT TYLER.

Establishment of the direct inhibiting activity of 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-anisylethanol (referred to as MER-25) on Arbacia egg development has led to an investigation of a related group of triphenylethanol derivatives and of several synthetic estrogens with structural similarities. MER-25, at a concentration of $2 \times 10^{-5}\%$ does not impair fertilizability of eggs, but subsequent cleavage is prevented or proceeds abnormally. Those eggs that divide are generally blocked in an abnormal blastula stage and remain intact for 36–48 hours before disintegrating. If treatment is initiated at stages up to the just-hatching blastula, development is blocked before gastrulation, the embryos again remaining intact and motile for a considerable time. Post-gastrulation stages are not visibly affected by treatment with MER-25. At the same concentration, the synthetic estrogens, stilbestrol and hexestrol, are cytotoxic to all embryos, pre- or post-gastrulation. When administered to unfertilized eggs, these compounds prevent subsequent fertilization. Unfertilized eggs, newly-fertilized eggs and developing zygotes (including well-formed plutei) become non-viable and cytolyze within several hours after exposure to these compounds. This appears to be a general cytotoxic effect, distinct from the blockage action exhibited by MER-25. The same concentration of a third synthetic estrogen, tri-(*p*-anisyl)-chloroethylene (TACE) has no inhibitory action on Arbacia development. The unsaturated parent compound, triphenylethylene, is likewise ineffective. Several derivatives of the latter, with side-chains bearing structural similarities to the diethylaminoethoxyphenyl group and with the 1-carbon hydroxyl substitution of MER-25, exhibit the blockage activity. The data suggest that the anti-zygotic activity of MER-25 is not related to its general structural similarity to some synthetic estrogens nor does the activity derive from the triphenylethylene moiety which provides the basic configuration for the compounds in this series.

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[1] Supported by the Population Council and by research grant (C-2302) from the National Cancer Institute, U.S.P.H.S.

A study of current orientation as a stimulus to schooling behavior in Menidia.

EVELYN SHAW.

Most fish, including *Menidia,* show a positive rheotaxis when placed in a moderate current flow. This response is found as early as hatching. Newly hatched *Menidia* immediately orient upstream and maintain a constant swimming speed within the current. It seemed possible that orientation into a current might be an effective stimulus for the development of the parallel pattern of swimming found among schooling fish. Orientation into the current could, for instance, accustom pre-schooling fish to seeing their species mates in certain visual patterns which would influence the fish in their mutual response in such a way that this familiar visual pattern would be maintained. To evaluate the influence of current flow on the development of schooling, fish were reared to a length of 15 mm. in bowls of still water. Under these conditions, schooling developed at the same age and with the same characteristic patterns found among fishes reared in a moderate current flow.

A study of visual attraction as a stimulus to schooling behavior in Menidia.

EVELYN SHAW.

Visual attraction as a primary stimulus for schooling has been reviewed by Morrow and Atz. In order to determine whether visual attraction is an important stimulus in the development of schooling in *Menidia,* experiments have been carried out by a technique which was described last year. This technique consists in presenting a freely-swimming fish to a fish of the same size enclosed in a narrow glass tube. The studies of last year on early schooling fish (about 12 mm. in length) have been extended to include fish of lengths varying from 5 to 16 mm. It was found that fish of 5, 6 or 7 mm. in length did not approach the enclosed fish. Fifty per cent of fish of 8 to 14 mm. in length approached and adopted a parallel orientation for brief periods (2 to 3 seconds). Eighty per cent of fish of 15–16 mm. in length oriented and swam parallel to the enclosed fish. This orientation was maintained for periods of time up to one minute during which the direction of swimming changed as many as seven times. In all cases, the freely-swimming fish did not respond to the enclosed fish immediately after presentation of the tube. The latency of response in fish of 8–14 mm. in length averaged 3½ minutes and in fish of 15–16 mm., 50 seconds. Contrary to our observations with this technique, it should be noted that freely-swimming fish of 5–7 mm. in length approach one another. They do not, however, orient with one another. Two of the possible explanations for this difference in behavior are (1) approach by fish, 5–7 mm. in length, is not relevant to schooling behavior, (2) that fish of this age require a response from a species mate which cannot be given by a fish which is restricted within a glass tube.

The occurrence of amicronucleate tetrahymenae as facultative parasites in embryos of the catfish Ameiurus.[1] CARL CASKEY SPEIDEL.

Ciliated protozoan parasites were found by P. B. Armstrong in a few embryos of the catfish *Ameiurus* collected from a fresh water pond in Falmouth, Massachusetts. When examined by us the living embryos were in the yolk-sac stage and the parasites were present especially, though not exclusively, in the developing central nervous system. Superficial observation at once indicated that the parasites were much like the tetrahymenae previously reported by us as facultative parasites in tadpoles of the toad *Bufo,* now classed as *Tetrahymena corlissi.* An important difference, however, was the absence of a micronucleus in the catfish parasites. The organisms were observed further in cultures in which the food consisted of excised pieces of tadpole tissues. Their details of structure as well as their methods of movement, feeding, reproduction, and invasion of tissues seemed quite like those of *T. corlissi.* Furthermore, like *T. corlissi* the catfish parasites were able to invade the tissues of living tadpoles rendered vulnerable by experimental lesions or by x-ray irradiation.

Thus, the catfish parasites appear to be a naturally occurring strain of amicronucleate tetrahymenae possibly to be classified as *T. corlissi.* Final determination of the species, however, must await a study of silver-stained specimens.

Radiation-induced variations in the micronucleus of Tetrahymena corlissi.[1] CARL CASKEY SPEIDEL.

As previously reported for two strains of *Tetrahymena corlissi* the micronucleus, a center rich in deoxyribonucleic acid, was eliminated by repeated severe x-ray treatments. A like result has now been obtained with a third strain which was found invading the tissues of tadpoles of *Pseudacris* which had been subjected to ultraviolet radiation. The tadpoles and the tetrahymenae of this strain were collected in a swamp near Charlottesville, Virginia.

Further studies have made it clear that marked micronuclear variations could be induced by single x-ray treatments of from 400–700 kr. Although a single dose of 500 kr killed the great majority of irradiated tetrahymenae, a few survived and multiplied. These gave rise to a progeny made up of individuals that differed with respect to micronuclear content. Such descendants were kept under observation for a year for each of two strains. The descendants of one strain (Woods Hole Strain W) exhibited the following micronuclear variations: a few had no micronucleus; a majority had a single micronucleus of reduced size (some, however, of normal size); some had 2 micronuclei; and a few had 3, 4, or 5 micronuclei. A clone culture derived from a single descendant gave rise to individuals with similar micronuclear variations. This result seemed to indicate that there had been a profound disturbance in the normal mechanism by which during binary fission each daughter cell received one micronucleus. The descendants of another strain (Thompson's Charlottesville Strain Th-X) after a dose of 500 kr were almost entirely without a micronucleus. A clone culture derived from one of these was made up of amicronucleate individuals only.

Micronuclear variations like those listed above were also induced in *Tetrahymena corlissi* by repeated ultraviolet radiation. This type of radiation, however, was much less effective than x-radiation in producing amicronucleate individuals.

Conduction in dogfish spiral-valve retractor and Phascolosoma proboscis retractor muscles. W. W. STEINBERGER AND C. LADD PROSSER.

The retractor of the spiral-valve is a sheet of non-striated muscle arranged in fasciculi with individual fibers 3 μ in diameter and 0.1 mm. long. Simultaneous recordings of mechanical contractions and external action potentials show in response to electrical stimuli a burst of spike-like action potentials preceding sustained contractions lasting several minutes. During the maintained tension electrical activity decreased (sometimes ceasing) only to increase (or reappear) with the onset of relaxation. The action potentials and the corresponding tension waves then decrease and cease. A quick short mechanical stretch is an adequate stimulus giving a similar response pattern. Action potential propagation is about 2 cm./sec. Methylene blue stain indicated the presence of nerve fibers but no evidence of nerve impulses prior to muscle response were seen when recording externally with high amplification. Resting potentials as high as 70 mV. were recorded with microelectrodes. Intracellular action potentials did not overshoot and are spike-like with a duration of approximately 0.2 second. The spiral-valve retractor muscle is similar to mammalian visceral muscle in that there is interfiber conduction independent of nerves and shows graded cellular potentials. When a region of the retractor was mechanically fixed propagation of action potentials occurred through the mechanical block showing conduction in the absence of mechanical pull.

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[1] This investigation was supported by a research grant (PHS RG-4326 C) from the National Institutes of Health, Public Health Service.

Previous evidence showed that unlike vertebrate smooth muscles the Phascolosoma proboscis retractor contracts entirely by nervous control. The muscle action potential shows fast and slow components conducted in different nerve fibers. Microelectrode records showed that many muscle fibers give both fast and slow potentials, while some give only one type. Many of the individual muscle fibers have, therefore, double innervation. Intracellular potentials are low (40 mV.) probably due to the microelectrode not sealing in the fibers (5 μ). Quick stretch failed to stimulate the proboscis retractor.

Enzymatic inactivation of chromatophorotropic principles from the fiddler crab, Uca. G. C. Stephens and J. P. Green.

Extracts of the green gland of *Uca* retained their chromatophorotropic activity for at least forty-eight hours provided the extract was boiled for ten minutes. By contrast, unboiled kidney extract was inactive twelve to twenty-four hours after preparation. This suggested that the green gland might possess the capacity to inactivate chromatophorotropins from other sources in the organism such as the sinus gland, thoracic cord and brain and circumesophageal connectives.

In order to test this possibility, extracts of these organs as well as of the green gland were prepared at suitable concentrations and boiled for ten minutes. Unboiled extracts of hepatopancreas, green gland, skeletal muscle and heart were prepared. Each unboiled extract was tested for its capacity to inactivate the chromatophorotropins from the four sources mentioned. Equal volumes of unboiled extract and each of the boiled extracts were mixed, streptomycin added, the mixture allowed to stand at room temperature for twelve to twenty-four hours, and then injected into previously prepared destalked assay animals. The extracts of skeletal muscle and heart were inactive. The previously reported presence of an inactivating material in the hepatopancreas was confirmed. In addition, inactivation of chromatophorotropic principles by unboiled kidney extract was observed.

Activity of trypsin and papain was also tested on boiled extracts of endocrine sources. Both proved effective in destroying chromatophorotropic activity though trypsin was effective only at very high concentrations (7%).

Hepatopancreas extracts are capable of digestion of gelatin but extracts of the green gland are not.

The sensitivity of these chromatophorotropins to trypsin and papain, together with their stability to boiling, suggests they may be polypeptide in nature. These observations further suggest that inactivation of extracts by the green gland may be more specific in character while inactivation by hepatopancreas may be a consequence of its general proteolytic capacity.

Chromatophorotropic principles of the green gland of the fiddler crab, Uca. G. C. Stephens, B. Guttman and J. P. Green.

Chromatophorotropic activity of the green glands of the fiddler crab, *Uca pugilator,* was measured by the following technique. Male donor and assay animals were prepared by removing the eyestalks, so that the melanophores became punctate and the guanophores dispersed. Green glands were removed from these animals and ground in sea water to make an extract with the concentration of 10 glands per 0.5 cc. of water. Five-hundredths cc. of this extract was then injected into each assay animal. The chromatophores were staged at intervals of ½, 1, 1½ and 2 hours after injection and their total excursion from the initial condition was used as a measure of activity of the extract.

Green glands of donor animals produced strong black expansion and white contraction; there is also some indication of a red concentrating element. As a control, various concentrations of ammonium chloride and urea in sea water were injected; these produced no chromatophore changes. Green gland extract shaken with Permutit to remove ammonia retained chromatophorotropic activity. A dilution curve was obtained showing chromatophorotropic activity as a function of extract concentrations.

An attempt was made to modify the chromatophorotropic activity of green gland extracts by injecting extracts of sinus gland or thoracic cord into donor animals and permitting them to respond before removal of their glands. The majority of such experiments produced a highly significant decrease in chromatophorotropic activity of the subsequently assayed green glands when compared with those of donors which did not receive extracts. However, other experiments failed to exhibit such a difference, particularly when the green gland activity of the uninjected donors was initially low. Some additional explanation is required to account for the variability encountered in the green gland extracts of both destalked and normal donor animals.

Studies on the effect of population size on the diurnal melanophore rhythm of the fiddler crab, Uca. G. C. STEPHENS, J. P. GREEN, B. GUTTMAN AND R. A. SCHINSKE.

Thirty-six *Uca pugnax* were isolated in individual glass jars and placed in a darkroom along with two pans of 25 animals. The melanophores of these animals were staged for forty-eight- to sixty-hour periods six times, providing a discontinuous record of the melanophore rhythm over a period of two months.

No maintained drift was evident in the time relations of the cycle in either group although departures as large as four hours from the originally established rhythm were observed. The previously reported decline in the amplitude of the rhythm was observed as an increasingly obvious effect. The over-all stability of such isolated animals with respect to time of dispersion of their chromatophores is indicated by the fact that at the the end of two months of isolation, only four of the 32 animals surviving had lapsed totally out of phase. The form and timing of the cycle of individual animals varied considerably from one day to the next, with differences as great as eight hours being observed in time of dispersion and contraction.

Numerous observations were made in an effort to determine whether this effect on amplitude was mediated through the release of some metabolite or other chemical agent. Isolated animals were subjected to periodic changes of the surrounding medium using extracts of endocrine glands, green glands and water recovered from pans containing groups of animals. The amplitude of the rhythm which they exhibited was then compared with that of suitable controls. Grouped animals were placed in running sea water and compared with controls at the same temperature maintained in standing water. Finally, isolated animals maintained in a common water supply were compared with totally isolated animals. Evidence for any chemical mediation of the amplitude effect was occasional and dubious so that one must conclude on negative grounds that physical contact between individuals is necessary and sufficient for maintenance of normal amplitude of the diurnal melanophore rhythm.

Relation of halogen position to physiological properties of mono-, di-, and trichlorophenoxyacetic acid. FREDERICK N. SUDAK, C. LLOYD CLAFF AND ALAN GREENBERG.

Previous experiments in our laboratory have shown that 2,4 dichlorophenoxyacetic acid (300 mg./kg.) rendered rats poikilothermic in relation to changes in ambient temperature. Animals treated with this compound were unable to increase their metabolism or maintain body temperature in a cold environment (7° C.). Conversely these animals could not lose body heat nor control their metabolism when exposed to warm (35° C.) air. The effect of 2,4,5 trichlorophenoxyacetic acid (300 mg./kg.) was similar to that of "2,4-D" on metabolic and body temperature responses to changes in ambient temperature. Phenoxyacetic acid, ortho-, and parachlorophenoxyacetic acid (300 mg./kg.) were without effect.

A bacteria-free inhibitor of regeneration in Tubularia. KENYON S. TWEEDELL.

Inhibition of regeneration in Tubularia can be produced by bacteria-free filtrates of the culture medium. The inhibitor was obtained from adult hydranths removed from the colony with portions of the cut stems attached. Fully mature individuals were avoided. The animals were washed in 5 washes of sterile sea water and placed in bacterial filtered sea water in a ratio of 3 animals/ml. The inhibitor was collected in a modified twin-spout Ehrlenmeyer flask. Rotation of the animals was accomplished by an aerator leading inside to the flask bottom. The temperature was maintained at 15° C. by means of a refrigerated bath to suppress bacterial activity. Every hour the collecting fluid was bled off through a side arm and immediately returned to the flask through a pressure-operated Seitz bacterial filter (type EK filter). Potential growth of any bacterial population was thus prevented. Harvest of the inhibitor water was taken at 12 or 18 hours. The resulting fluid was clear and odorless with a lower bacterial count than that of normal sea water.

The inhibitor water was applied to freshly amputated stems kept in a water bath at 15° C. After 70 hours, all control stems had regenerated. The stems in 12-hour inhibitor filtrates never emerged. The inhibitor often affected differentiation but did not restrict internal circulation or movement of the coenosarc. Often, small bulbs of coenosarc moved beyond the cut ends of the stems or occasional abortive pinch stages (constriction without differentiation) were encountered. Complete cessation of activity was obtained with the 18-hour filtrates.

Stems treated with either 12- or 18-hour bacterial filtrated inhibitor and kept at 21° C. regenerated at a greater rate but gave identical results.

Production of S^{35}-labelled fertilizin in eggs of Arbacia punctulata.[1] ALBERT TYLER AND RALPH R. HATHAWAY.

The fertilizins of eggs of sea urchins are glycoproteins containing considerable sulfate which is probably ester-linked. It seemed likely, then, that the administration of S^{35} in the form of inorganic sulfate during the period of ripening of oocytes would lead to its incorporation in the fertilizin. This expectation has been realized.

Ripe females were induced to shed their eggs by means of KCl-injection. They were then each given a series of three, alternate-day, 50-microcurie injections of an S^{35}-labelled sulfate solution. The animals were kept in non-circulating sea water (six per 3 liters), which was changed before each injection. The day after the last injection of the series the animals were placed in running sea water overnight. The eggs were then collected, by the usual KCl-injection procedure, in the cold, and washed several times with cold sea water. The washings showed no appreciable content of S^{35}. The fertilizin was then dissolved off the eggs, by lowering the pH of the suspension to about 3.5 to 4, and found to be highly radioactive. A sample of fertilizin (*ca.* 15 mg.) from about 1 ml. (packed volume) of eggs gave counts (gas-flow type counter) of about 60,000 per minute, corresponding roughly to an activity of 0.003 microcurie per mg. fertilizin, about one-fourth of which is sulfate. Four series of injections were run with 12 to 18 animals, most of which were used continuously and produced fair yields (*ca.* 1 to 4 ml.) of eggs after each series, with approximately the above mentioned content of S^{35} in their fertilizin.

The influence of protoporphyrin-nitroresorcinol and other phenols on x-radiation sensitivity of Paramecium caudatum. RALPH WICHTERMAN,[2] HARVEY SOLOMON AND FRANK H. J. FIGGE.[3]

We previously reported that when phenol was added to clonal cultures of paramecia before irradiation, the LD/50 dose fell from 340 kr to less than 18 kr. A trinitro-resorcin protoporphyrin complex was prepared to determine its effect on radiation sensitivity of *Paramecium caudatum* and later to test the tendency of the complex to accumulate in tumors. At the same time, trinitroresorcinol was tested in addition to a series of other tri-, di-, and mono-hydric phenols. The porphyrin-resorcinol compound increased the radiation sensitivity of paramecia.

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[1] Supported by a research grant (C-2302) from the National Cancer Institute, U. S. Public Health Service and by AEC Contract AT(30-1)-1343 to the Marine Biological Laboratory.

[2] Part of a project aided by a contract between the Office of Naval Research, and Temple University (NR 104-475) and the Committee on Research, Temple Univ.

[3] Supported by the American Cancer Society (Maryland Division), Grant. No. CY3580 (C1) from the Cancer Division of USPHS, and the Anna Fuller Fund.

Paramecia from clear lettuce medium were placed in phenol solutions ranging from 1 to 10,000 to 1 in 50,000 and then exposed to 50 kr of x-irradiation in closed one-ml. Lucite chambers completely free of air pockets. Immediately after irradiation, the paramecia were exposed to the air. The organisms died within one-half to two hours (depending upon the concentration and potency of the chemical) in chambers containing phenol-, ortho-, meta-, and para-nitrophenol, resorcinol, catechol, and phlorglucinol. Neither the irradiated controls, nor the controls treated with the above chemicals were affected. Non-irradiated hydroquinone and pyrogallol solutions exposed to air were so toxic that paramecia did not survive a 1 to 500,000 concentration.

These experiments indicate that the phenol in the culture medium is probably converted into hydroquinone, resorcinol, catechol, pyrogallol, and similar partially oxidized phenols which result from reaction with the peroxides formed during the irradiation. The fact that the paramecia irradiated in phenol solutions do not die until the solution is exposed to air indicates that these slightly toxic irradiation products require autoxidation in air to exhibit maximum toxicity.

Effect of temperature on circulation in Cistenides. CHARLES G. WILBER.

The trumpet worm, *Cistenides,* has a major pulsating blood vessel which is clearly visible through the body wall of the living animal. Observations of the rate of pulsation of the vessel can be made readily by examining the animal under good illumination after it is removed from its cone-shaped tube. Approximately 50 of these worms were exposed to aerated sea water at various temperatures above 20° C. The rate of pulsation of the blood vessel was observed under low magnification (10 ×) and recorded. The average rate over a 30-minute period at a given temperature was calculated and plotted. If the logarithm of the rate in beats per minute is plotted against the water temperature in ° C., a good straight line is obtained between 20° C. and 31° C. Between 31° C. and 35° C. the slope of the line is decreased. Above 35° C. the animal shows obvious distress and the rate of pulsation of the blood vessel becomes very irregular. The response of the heart to temperatures below 20° C. is now under study but adequate data are not yet available for report. Q_{10} value between 20° C. and 30° C. is 3; between 31° C. and 35° C., approximately 2. There is some indication that lysergic acid diethylamide, yohimbine and other drugs influence the rate of pulsation of the vessel in question. Such drug studies are now in progress.

Partial support of this work came from a National Science Foundation Grant.

The morphology of the copepod Congericola pallida from the gills of Conger vulgaris taken at Woods Hole. CHARLES H. WILLEY.

Congericola pallida Van Beneden 1854 is found infrequently along the European coast but has not been hitherto reported from American waters. One large conger among eleven examined harbored about fifty mature female specimens of the copepod. No males were encountered. A few scattered accounts of the European forms have been published, but as pointed out by Wilson in a monograph on the Dichelesthiidae, a dearth of information exists on the morphology of the species. The thoracic appendages are of importance taxonomically and have been differently described by different investigators. Examination of living and preserved material reveals that there are four pairs and all are biramose. The endopodites and exopodites of the first pair are two-jointed and those of the second and third pairs are three-jointed. The rami of the fourth pair are larger and consist of but a single segment. Differences reported in the width of certain thoracic segments are explained by variation in the degree of antero-posterior contraction of the body. Longitudinally contracted individuals exhibit a third thoracic segment a little narrower than the second while in fully extended specimens they are essentially the same width. The structures of the internal organs, studied from whole mounts and sections, show no essential differences when compared with other members of the Family Dichelesthiidae. It is concluded that the present material is specifically identical with the European form.

Carbon monoxide in the float of Physalia. JONATHAN B. WITTENBERG.

At the turn of the century Schloesing and Richard found the gases contained in the float of the portuguese-man-of-war, Physalia, to be essentially similar to air in their content of argon, nitrogen and oxygen. In addition to these we have now found a fourth component, carbon monoxide. Individuals collected at Woods Hole contained in the float gases amounts of carbon monoxide varying from traces to eight per cent of the total gas. The majority of individuals contained from one to five per cent.

Carbon monoxide was determined volumetrically by reaction with a solution of cuprous chloride in ammonium chloride, and was identified by a characteristic color reaction catalyzed by palladium chloride, and by the characteristic carboxyhemoglobin spectrum exhibited by blood equilibrated with the float gases. Carbon monoxide accounted for all of the combustible gas present.

Although carbon monoxide is known to occur in the air spaces of some marine algae, to the author's knowledge the present finding represents the second discovered occurrence of carbon monoxide in animal tissues (the first being its origin during the degradation of hemoglobin).

Glucagon and blood glucose in Lophius piscatorius.[1] PAUL A. WRIGHT.

Since the realization that bullfrogs had very low blood glucose values, or in 28% of the cases glucose absence, we have been interested to know whether any other vertebrate was also anomalous in this respect. As tested by the same modified Nelson procedure used for the frog, marine fishes showed average glucose values as follows (number of animals in parentheses): *Mustelus canis*, 87 mg. % (8), *Raia diaphanes*, 78 mg. % (4), *Dasyatis centroura*, 54 mg. % (7), *Serranus atrarius*, 57 mg. % (8), *Prionotus strigatus*, 66 mg. % (6), and *Tautoga onitis*, 59 mg. % (12). *Lophius piscatorius* (13), in contrast, gave an average value of only 7.6 mg. %, with a high of 15 mg. % and two animals with glucose absence. Intra-arterial administration of crystalline glucagon (10 μg./kg. and 100 μg./kg.) failed to induce any detectable hyperglycemia within 40 minutes after injection. Failure to respond to glucagon puts *Lophius* in a category with the salamander, *Taricha torosa*, as reported by Miller and Wurster. Bullfrogs, on the other hand, develop hyperglycemia rapidly in response to glucagon injection.

Extracts of the principal islet of *Lophius*, prepared according to the method of Sutherland and de Duve, induced a consistent hyperglycemia (40 mg. % in 40 minutes) after intra-arterial injection in bullfrogs. Such extracts were still potent after incubation with 0.1% cysteine at 38° C. for 4 hours, thus destroying any possible epinephrine or insulin contamination, and are therefore presumed to contain glucagon.

LALOR FELLOWSHIP REPORTS

The osmotic behavior of marine oocyte nuclei. CLIFFORD V. HARDING.

Osmotic properties of nuclei have been reported for intact amphibian and marine oocytes and for isolated amphibian germinal vesicles. Unlike amphibian germinal vesicles, however, starfish oocyte nuclei do not swell upon isolation into simple salt solutions or glass-distilled water. On the other hand, isolated germinal vesicles of starfish and *Spisula*, as well as those of *Hydroides* (Ashton), do change volume with change in *colloid* osmotic pressure of the surrounding medium. For example, they showed a decrease in diameter when placed in solutions of polyvinylpyrrolidone (PVP, mol. weight, 40,000) of 5% or greater, made up either in 0.53 *M* KCl or glass-distilled water. This is similar to the results obtained previously with amphibian oocytes. It is possible to demonstrate this also in starfish nuclei which remain within cytolyzed oocytes. Oocytes which had been cytolyzed showed no increase in nuclear diameter. They did, in fact, show a decrease (Feldherr). However, when these cytolyzed cells were placed in 5% PVP in glass-distilled water, there was a rapid shriveling of the nuclei. In some cases, there was a return to spherical shape of the nuclei within 30 minutes, indicating the possibility that the PVP may be penetrating these nuclei. Similar experiments were carried out in which the cytolyzed oocytes were transferred to 0.53 *M* KCl before being placed in the PVP, which was also made up in 0.53 *M* KCl. These experiments were complicated by the fact that the cytolyzed cells themselves decreased markedly in volume when placed in the KCl-PVP solution, obscuring any changes in the nuclei. The differences observed between amphibian and starfish nuclei may represent inherent differences in the properties of the nuclei, or, perhaps, differences in the extent of injury sustained by the nuclei as a result of the process of isolation.

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[1] Supported by grants from the U. S. Public Health Service, A-1280 (C) and the Horace H. Rackham Fund, University of Michigan.

Uptake of tritium-labeled thymidine by Arbacia eggs and embryos. CLIFFORD V. HARDING AND WALTER L. HUGHES.

Investigations were carried out to measure the uptake of tritium-labeled thymidine into *Arbacia punctulata* eggs and embryos, and to determine if this uptake resulted in any inhibition of development. Unfertilized eggs and embryos at various stages of development (early cleavage through gastrulation) were incubated for different periods of time in low concentrations of tritium-labeled thymidine (3–8 microcuries per culture; culture volumes varied from 20 to 150 ml.). Samples of eggs, embryos and supernatant culture media were preserved in absolute alcohol. The samples were washed further with absolute alcohol or 1 per cent NaCl, dried, digested in H_2SO_4—HNO_3, and counted in a liquid scintillation counter. An internal standard was used to correct for quenching. The samples of embryos consistently showed an uptake of tritium into the insoluble phase, and they showed increased counts with increased time of incubation. The activities varied from 2 to 20 disintegrations per minute per egg, depending primarily on the time of incubation. Uptake occurred as early as 106 minutes after fertilization, which, at 20° C. is just after the second cleavage. Unfertilized eggs, on the other hand, showed no concentration of tritium activity. It is suggestive that the activity determined in the developing embryos is truly built into DNA; however, it would be important to isolate and determine the specific activity of the DNA in order to establish this. There was no noticeable inhibition of development in the tritiated eggs. This is perhaps not surprising in view of the comparatively large amount of tritium necessary to inhibit the multiplication of cells in tissue culture (Painter, Drew and Hughes, 1958). It would be of interest to extend the studies on *Arbacia,* using higher concentrations of tritium-labeled thymidine, and determining the distribution of insoluble activity within the egg by radioautography.

Active transport of oxygen. JONATHAN B. WITTENBERG.

Two structures participate in the transport of oxygen gas from the circulating blood into the swimbladder of fishes. These are the gas gland and a vascular counter-current exchanger, the rete mirabile, supplying blood to that gland. We were emboldened by Ruud's description of certain antarctic fishes, totally lacking hemoglobin, to attempt experimentally to reduce to low levels the circulating oxyhemoglobin of locally available fishes, and in that way study the function of the gas gland uncomplicated by the action of the rete.

Toadfish (*Opsanus tau*) were made to breathe gas mixtures containing varying amounts of carbon monoxide (0.2 to 35 per cent carbon monoxide, 50 per cent oxygen, balance nitrogen), which especially in the higher ranges should be sufficient to convert essentially all of the blood hemoglobin to carboxyhemoglobin. These animals continued to secrete oxygen into the swimbladder, thus proving that the gas gland cells are capable of transporting oxygen from the blood plasma into the swimbladder. In addition to oxygen the secreted gas contained a substantial concentration of carbon monoxide. Since animals breathing 0.2 and 5 per cent carbon monoxide secreted gas mixtures containing 10 and 30 per cent carbon monoxide, respectively, carbon monoxide transport must also be considered to be an active process.

From the compositions of the gas mixtures breathed and secreted, the relative affinity of the glandular oxygen transporting system for oxygen and for carbon monoxide may be approximated. Over the entire range of carbon monoxide concentrations studied, the affinity for carbon monoxide was found to be 3 to 8 times as great as the affinity for oxygen.

It is reasonable to assume that an iron-heme protein is implicated in the active transport of oxygen.

Vol. 115, No. 3 December, 1958

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

SOME ASPECTS OF REPRODUCTIVE BIOLOGY IN THE FRESH-WATER TRICLAD TURBELLARIAN, CURA FOREMANII[1]

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Apparently alone among the fresh-water triclad turbellarians for which the details of reproduction are known, *Cura foremanii* is capable of sexual reproduction under conditions which prevent the occurrence of copulation between two individuals. This was established for a Rhode Island strain of this species by Anderson (1952a), who isolated individuals on the day of hatching and found that upon reaching sexual maturity each worm deposited numerous cocoons, a large majority of which were usually fertile and produced normal juveniles. Furthermore, it was established that reproduction could continue in this way for long periods without apparent detriment to the viability or reproductive capacity of the strain. In one series of observations, terminated after 20 months, a pedigreed line of worms produced 9 successive generations, each generation represented by one individual isolated as soon as it had hatched and maintained in isolation until its first offspring appeared (Anderson, 1952b and subsequent unpublished observations). As these worms were apparently capable of maintaining reproductive activities indefinitely without opportunity for copulation, and as we had never observed copulation in mass or paired cultures of *Cura,* it was suggested that copulation between individuals might not be a normal prelude to sexual reproduction in this species. Kenk (1935) has reported that *Cura foremanii* lacks a true copulatory bursa, an organ invariably present in copulating species; in *Cura* it is represented only by its stalk, which connects with one of the medial branches of the intestine and forms a genito-intestinal canal. These facts support the suspicion that *Cura* must reproduce without copulation.

In the initial report of the unique reproductive habits of *Cura foremanii,* it was impossible to state whether reproduction in isolated individuals involved fertilization of eggs by spermatozoa from the same worm or parthenogenetic development of unfertilized eggs. Neither parthenogenesis nor self-fertilization had been reported in other fresh-water triclads by previous authors, and no direct evidence favoring either possibility was obtained from our observations.

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[1] Referred to in previous publications as *Curtisia foremanii;* best taxonomic account is that of Kenk (1935). The name *Curtisia* (given this worm in 1916 by von Graff, in honor of Prof. W. C. Curtis) is preoccupied; Strand (1942) has proposed the substitution of *Cura* for *Curtisia.*

The apparently simple question of the nature of the reproductive process in this species has proved surprisingly difficult to answer unequivocally. Evidence bearing on the central problem has been sought by attacking several subsidiary questions, such as the following:

1) Is sexual reproduction in isolation characteristic of the species as a whole, or is it limited to the stock with which the original work was done?

2) In sexually mature and reproductive individuals, reared in isolation, are spermatozoa present in the seminal receptacles (the anterior extremities of the ovovitelline ducts, adjacent to the ovaries), as they were reported to be by Curtis (1900) and by Stevens (1904) in animals taken from the wild and from mass cultures?

3) If spermatozoa are found here in isolated individuals, when do they first arrive at the seminal receptacles? Is there any correspondence between the time of first appearance of spermatozoa at the ovaries and the time of deposition of first cocoons by isolated worms just attaining sexual maturity? Or can an individual deposit fertile cocoons before spermatozoa first appear in its seminal receptacles?

4) Do eggs deposited in cocoons by isolated individuals show any evidence of having been penetrated by spermatozoa?

5) Do spermatozoa ever appear in the bursa stalk (genito-intestinal canal) of mature worms taken from mass cultures? That is, is there evidence that what remains of the copulatory bursa is used in copulation in mass cultures? If spermatozoa are found here, how do worms from mass cultures compare in this respect with mature and reproductive individuals reared in isolation?

This paper reports the results of investigations designed to provide answers to these and related questions and discusses the relationship between this information and the major problem of the nature of the reproductive process in *Cura foremanii.*

Materials and Methods

The worms on which these observations have been made constitute several stocks of diverse geographical origins. One stock represents descendants of the specimens collected in 1950 from a stream near Geneva, Rhode Island, in which reproduction in isolation was originally described. Another group of worms originated with several specimens collected in Cascadilla Creek, near Ithaca, New York. A third stock consists of offspring of several mature specimens collected from a stream in the Adirondacks, near Warrensburg, New York, by Neal R. Foster. The fourth strain was purchased from a biological supply house, having been collected in Powder Mill Park, near Rochester, New York.

The methods used in maintaining these stocks are essentially those described earlier (Anderson, 1952a), with some modifications. All worms have for the past few years been kept in a constant-temperature room under continuous illumination. Temperatures have varied over long periods between 21° and 22.5° C.; illumination is provided by two 40-watt fluorescent tubes about four feet above the table on which the cultures are kept. Specimens have been maintained both in mass cultures and in isolation. Each of the isolated worms is kept in about

50 ml. of water in a 4-ounce jar. Small mass cultures (6 to 12 individuals) are kept in larger amounts of water in identical jars; larger mass cultures of several dozen individuals occupy wide-mouth jars containing approximately 200 ml. of water. All culture jars are tightly closed with metal screw caps.

The sources and types of water used for the cultures have been subject to considerable variation through the past several years. When the Rhode Island strain was first transferred to Ithaca, and for some years subsequently, these worms and the locally-collected stocks were kept in water obtained from Cascadilla Creek at the Cornell University fish hatchery. In 1957 all stocks were changed to a "modified tap water" suggested by Loomis and Lenhoff (1956), prepared by adding 50 mg./L each of disodium ethylenediaminetetraacetate and $CaCl_2$ to tap water. For the past few months we have used unmodified Ithaca tap water without apparent unfavorable effects on the cultures.

All worms are fed regularly, once each week. Isolated worms are given bits of fresh mouse-liver, while the mass cultures are fed liver and such other organs as heart, spleen, and kidneys of freshly-killed mice. Food is allowed to remain in the jars for at least several hours and sometimes overnight, after which the remnants are removed and the water changed.

The worms are examined carefully at one- or two-day intervals. For each of the isolated worms, records are kept of the dates of deposition and hatching of all cocoons, and of the number of young produced. Juvenile worms are removed from these cultures as soon as possible after hatching; they are either transferred to mass cultures or set up individually in isolation to maintain and extend selected pedigreed stocks. Following the scheme previously described (Anderson, 1952a), each such isolated juvenile receives a code designation denoting its generation and its ancestry.

For the specific purposes of the present investigation it was necessary to prepare serial sections of mature worms from both mass and isolation cultures, as well as of a series of developing juveniles sacrificed at weekly intervals from the time of hatching to the attainment of sexual maturity. Specimens were anesthetized by placing them in a solution of MS-222,[2] 1:1500 by weight, as suggested by Manner (1957); when relaxed, the worms were flooded with either Zenker-acetic or Helly's fluid. They were kept extended and moderately flattened during fixation by compressing them gently under a coverslip. Following standard procedures, the specimens were imbedded in Tissuemat; serial sections were prepared at 7 μ, mounted, and stained in Harris' hematoxylin. Several freshly deposited cocoons were also fixed, imbedded, sectioned, and similarly stained, to provide material for determination of the nuclear condition of the eggs they contained.

OBSERVATIONS AND DISCUSSION

The ability of *Cura foremanii* to carry on active sexual reproduction in isolation is not limited to the Rhode Island strain in which it was originally described. The other three stocks with which we have worked, collected from various localities in central and northern New York, have all proved capable of the same reproductive behavior. It therefore seems reasonable to conclude that this kind of reproduction, whatever the details of the process may be, is a general characteristic of the species.

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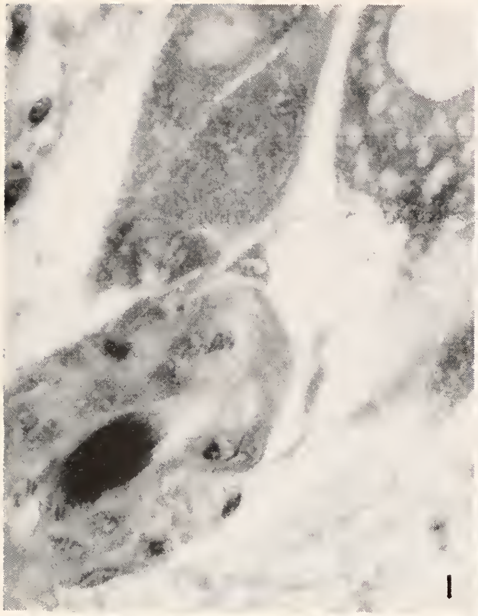
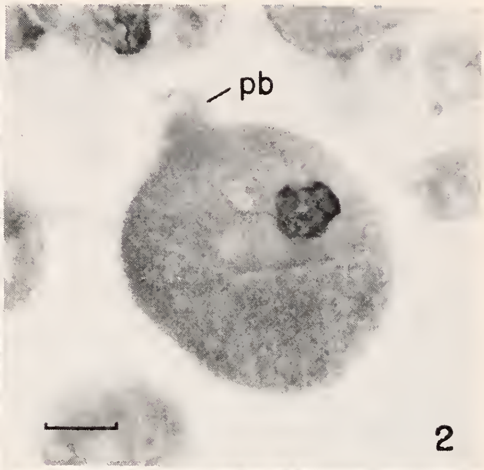
[2] A supply of this reagent was generously provided by Dr. Perry W. Gilbert. In our experience, exposure of planarians to MS-222 at this concentration almost invariably induces eversion of the proboscis, and the animal relaxes in this condition.

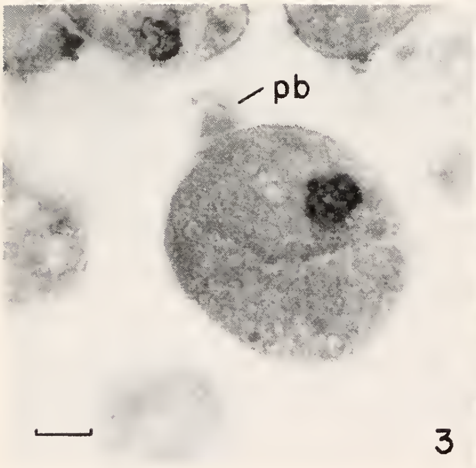
In all mature specimens of which sections have been examined, masses of spermatozoa have been found in the seminal receptacles. Figure 1 is presented as typical. This section is from an adult worm killed in the act of releasing a finished cocoon from its genital atrium. As the figure shows, the ovaries contain developing eggs in various stages of maturity, and the seminal receptacles are occupied by masses of spermatozoa. It is to be emphasized that in view of the anatomical relationships of the reproductive systems in fresh-water triclads, these spermatozoa could have reached the seminal receptacles only by moving anteriorly in the ovovitelline ducts from the genital atrium (see Curtis, 1900, and Kenk, 1935, for anatomical details of the reproductive system of *Cura*). Furthermore, since this individual had been maintained in isolation since the day it hatched, these spermatozoa could have originated only in its own testes and must have been emitted through its penis; spermatozoa are never found wandering about in the mesenchyme but are always restricted to the genital ducts.

All our observations indicate convincingly that in fully mature, actively reproducing specimens of *Cura* the seminal receptacles are invariably packed with spermatozoa; in isolated individuals these can have come only from the testes of the same animal. It is of interest to determine whether in young worms, just reaching sexual maturity, cocoons can be deposited before spermatozoa are produced and reach the seminal receptacles, or whether spermatozoa are already present in the seminal receptacles at the time of deposition of the first cocoon. Table I lists the ages at which 28 individuals of diverse origins, reared in isolation under constant conditions of temperature, light, and feeding, deposited their first cocoons. There is considerable variation, not apparently correlated with strain differences or any other obvious factors; the mean age at sexual maturity for this group of worms is 57.6 ± 2.5 days. Among these specimens, as among large numbers of other isolated individuals for which records have been kept, 40 days is the minimum age at sexual maturity; in all our experience with these worms, no isolated individual has ever deposited its first cocoon before about six weeks from its day of hatching.

The problem now becomes one of determining whether there is any correspondence between this earliest first-cocoon record and the time of arrival of spermatozoa in the seminal receptacles. Our study of this aspect of the problem involved the preparation and study of sections of a series of individuals of known ages, at weekly intervals from one week to seven weeks after the day of emergence from the cocoon. This afforded an opportunity for a general study of the development of both male and female systems, but since the details of such a study are not germane to the present problem only a summary of the principal events will be given. Several individuals of each age were studied, and there was some variation in the stage of development reached by different animals of the same age. Those showing the most rapid rate of development will be described, as they probably represent the group that would have shown earlier-than-average deposition of first cocoons. *At two weeks*, early ovaries and testes are recognizable, containing chiefly gonia and early gametocytes. *At three weeks*, the gonads are







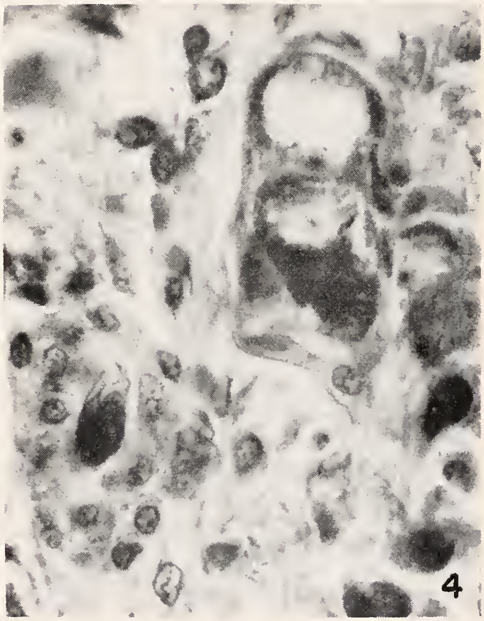


PLATE I

All figures are photomicrographs of sections cut at 7 μ and stained in Harris' hematoxylin. The scales represent 10 microns; that given for Figure 3 indicates magnification for Figures 1 and 4 also.

FIGURE 1. Portion of ovary and seminal receptacle of a mature individual, reared in isolation, fixed at the moment of deposition of a finished cocoon. At upper right, developing eggs in various stages; at lower left, mass of spermatozoa in seminal receptacle.

FIGURE 2. Fertilized egg in a cocoon, surrounded by yolk cells. Second polar body (pb) has been emitted; note vesicular female pronucleus and darkly-stained sperm nucleus.

FIGURE 3. Same section as that shown in Figure 2, at a slightly different focus, showing chromosomes in polar body (pb).

FIGURE 4. Portion of ovary and seminal receptacle of an individual reared in isolation for five weeks from the day of hatching. Note developing eggs in ovary (upper right) and mass of spermatozoa already present in seminal receptacle (lower left) in this animal which is just approaching sexual maturity.

TABLE I

Time required for attainment of sexual maturity at 22.5° C.

Worm	Date hatched	Date of 1st cocoon	Days to 1st cocoon
V9-1a-1a	1/8	2/17	40
T3-9a	1/7	2/17	41
V4-1a	10/9	11/19	42
V4-1b	10/9	11/19	42
*AD-9	10/24	12/10	47
*AD-5	10/24	12/11	48
V9-1a	11/8	12/26	49
T6-8a	1/8	2/28	51
*AD-8	10/24	12/14	51
†W2-1b	2/11	4/4	52
T10-11a	12/28	2/18	52
P8-1a	11/4	12/26	52
V9-12a	12/20	2/11	53
T10-11a-3a	3/20	5/12	53
*AD-3-2b	12/9	1/31	53
V10-1a	11/13	1/8	56
T10-1a	11/1	12/28	57
*AD-9-3a	1/7	3/3	57
†W2-1a	2/11	4/10	58
V6-1a	11/1	12/30	59
*AD-3-2a	12/9	2/7	60
P6-1a-11a	3/31	5/31	61
V4-1b-1a	11/29	2/7	71
V2-1a	11/11	1/22	72
V10-10a	12/20	3/11	81
T10-1a-1a	1/8	4/2	84
P6-1a	11/4	1/29	86
V4-1a-7a	1/2	3/29	86

* Adirondacks strain.
† Rochester strain.
Unmarked individuals are of Rhode Island or Ithaca strains.

larger and contain more advanced stages in gametogenesis; in addition, the earliest signs of development of the posterior genital complex are found at this age. *At four weeks,* large oöcytes are present in the ovaries, and the testes contain what appear to be well-developed spermatozoa. The genital atrium and associated structures are in an advanced state of development, but there are no signs that the ovovitelline ducts and the ductus deferentes are complete. *At five weeks,* the most advanced animal studied showed fully developed ovaries containing eggs well on the way to maturity; the testes were packed with mature spermatozoa; the genital complex posterior to the pharynx was completely formed, and sperm ducts and oviducts were present. Most significantly, at the age of 35 days from hatching, this individual's seminal receptacles were filled with spermatozoa. A portion of a section of this worm, showing some of these features, is presented as Figure 4. All individuals studied *at six and seven weeks* were in similarly advanced stages of sexual maturity, although none had deposited its first cocoon before fixation; in all, the seminal receptacles were filled with spermatozoa. The results of these studies are consistent with the conclusion that spermatozoa are always present in the seminal receptacles before the individual deposits its first cocoon, and thus that all eggs leaving the ovaries, even those first produced by an individual just reaching sexual maturity, must pass through a cloud of spermatozoa before reaching the genital atrium for inclusion in a cocoon.

Stevens (1904), in her studies of reproduction and early development in wild and mass-cultured specimens of *Cura foremanii* (referred to in her paper as *Planaria simplissima*, following Curtis' identification), reports that the first maturation division of oögenesis takes place within the ovary, and that the secondary oöcytes released into the seminal receptacles are then penetrated by the spermatozoa. The second maturation division occurs as the cocoon is being deposited, or very shortly thereafter, the sperm nucleus remaining quiescent until after the emission of the second polar body. We have now determined that in isolated individuals, just as in the cases reported by Stevens, spermatozoa are always present in the seminal receptacles after the attainment of sexual maturity. The problem remaining is to determine whether in these isolated individuals the secondary oöcytes passing through the seminal receptacles are penetrated by spermatozoa, or whether they remain unfertilized and develop parthenogenetically.

For information on this point we have turned to a study of the contents of freshly deposited cocoons. Each cocoon contains four to six eggs, extremely difficult to locate among the myriads of yolk cells with which they are surrounded. We were unsuccessful in our attempts to identify eggs in squash preparations of fresh cocoons but were able to locate several, in various stages of development, in serial sections. One of these eggs is shown in Figures 2 and 3; these are photographs of the same section, taken at slightly different levels of focus, showing the condition of the egg shortly after the formation of the second polar body. This cell contains, in addition to the vesicular female pronucleus, a densely staining mass which clearly represents the quiescent sperm nucleus (compare with Stevens' figures, 1904). Needless to say, a section such as this is exceptional; but there is no reason for believing that the condition it reveals is anything but typical. On the basis of such evidence we are led to conclude that the eggs of isolated individuals are fertilized, as they pass through the seminal receptacles, by spermatozoa from the testes of the same animal.

Although we may be confident that self-fertilization is the rule in isolated individuals, we have as yet no conclusive evidence as to whether reproduction in mass cultures involves copulation. Kenk (1935), reporting the absence of a copulatory bursa in *Cura*, nevertheless assumed (on what grounds we do not know) that copulation occurs: "In copulation, the relatively small penis is inserted into the posterior part of the bursa stalk 'vagina' of the co-copulant" (p. 82). In our own investigations, three individuals taken from mass cultures were fixed, sectioned, and stained, and their bursal stalks were carefully searched for the presence of spermatozoa. In only one of these were traces of spermatozoa found, but spermatozoa were found also in the bursal stalks of worms reared in isolation, in about the same proportion of cases. This is not a reliable kind of evidence, for two reasons. If copulation does occur between individuals, we do not know how long spermatozoa remain in the bursal stalk "vagina" before migrating into the seminal receptacles; those animals from mass cultures in which the stalks were empty might simply represent cases in which this migration had been completed. Furthermore, there is no certainty that spermatozoa might not be deposited in the bursal stalk by the penis of the same individual, or might wander into it seeking the openings of the ovovitelline ducts; in the isolated worms, spermatozoa must have reached the stalk in some such manner. Since the stalk forms a genito-intestinal connection, functioning, as Kenk suggests, to carry off "superfluous" sperm to the intestine for digestion, the spermatozoa found in the stalks of our isolated worms may simply be on their way to this fate.

The evidence bearing on the question of copulation in these worms is indirect and inadequate, but it seems a significant fact that the only species in which sexual reproduction is known to occur in the absence of copulation is also a species in which the copulatory bursa has been lost (or never developed at all) and is represented only by a genito-intestinal canal. To settle the question we need, simply, to observe two individuals in the act of copulation; failing this, we need a fertile individual showing some distinct heritable peculiarity of color or form which could be followed through breeding experiments. In our seven years' experience with this species we have observed neither copulation nor a distinctively-marked individual; this is an extremely uniform species. Exposure of large numbers of the worms to x-radiation might produce a mutation which could serve as a genetic marker. Such evidence as we now have, however, indicates the possibility, if not the probability, that *Cura foremanii,* which in isolation can substitute self-fertilization for copulation, may in fact have eliminated copulation as a normal feature of its reproductive processes.

Summary and Conclusions

A careful re-examination of the details of sexual reproduction in isolated individuals of the triclad species *Cura foremanii* has been carried out, designed particularly to determine whether this reproduction involves self-fertilization or simply the parthenogenetic development of unfertilized eggs. The following conclusions have been reached:

1) Without apparent detriment to the species, sexual reproduction can continue for many generations, and evidently indefinitely, under conditions of isolation which prevent the occurrence of copulation between individuals. This is true of four different stocks of the species, of diverse geographical origin.

2) All individuals examined during active reproduction showed masses of spermatozoa occupying the seminal receptacles through which the eggs must pass in moving from the ovaries to the genital atrium.

3) Studies of the development of the reproductive systems in immature isolated worms of known ages reveal that spermatozoa are mature and have already migrated to the seminal receptacles as early as 35 days after the emergence of the individual from its cocoon.

4) Under constant conditions, isolated individuals deposit their first cocoons some time between the 40th and the 86th day after emergence; thus, the eggs in even these first cocoons have always been exposed to spermatozoa in the seminal receptacles.

5) During this exposure to spermatozoa, the eggs are penetrated by them; eggs have been observed in freshly deposited cocoons showing second polar bodies

and vesicular female pronuclei, as well as distinctly staining sperm nuclei. Development of these self-fertilized eggs is presumably normal.

6) It is thus clear that sexual reproduction in isolated individuals of *Cura foremanii* involves self-fertilization and not parthenogenesis.

One tantalizing question remains unanswered: whether copulation ever occurs in *Cura,* even among individuals in mass cultures. This species lacks a copulatory bursa, possessing only the bursa stalk which serves as a genito-intestinal canal. Copulation has not been observed during our experience with this species, and clearly from the standpoint of normal reproduction and development the process is completely superfluous. Breeding experiments with marked individuals might settle the question; until these can be devised, it seems probable that *Cura foremanii* may have dispensed with copulation as a feature of its sexual reproductive processes.

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FORM-STABILITY OF CILIATES IN RELATION TO PRESSURE AND TEMPERATURE[1]

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The first use of hydrostatic pressure as an experimental parameter in biological research is credited to Regnard (1884a, 1884b, 1884c) and Certes (1884), working independently. Both of these early workers were impressed by the variety of living forms that had been recovered by the deep-sea dredging expedition of the *Talisman* in 1882–1883. Regnard, particularly, became interested in the effects of pressure *per se*. He eliminated changes in the gaseous equilibria by applying the pressure directly to the aqueous medium and he studied the effects of pressures ranging up to 1000 atmospheres on a wide variety of small aquatic organisms. In the present connection, he observed that various ciliates, crowded in stagnant water and subjected to pressures of 600 to 1000 atmospheres for 10 minutes, became immobile and distended, and that ciliary movement stopped. Within two hours after decompression many of the organisms seemed to have recovered completely.

Ebbecke (1935, 1936) described the effect of pressure on paramecia. Exposure to pressures of 500 atmospheres for periods of from 10 to 30 minutes mainly effected a change in the shape of the ciliates, *i.e.*, their bodies became more spheroidal. At pressures of from 800 to 1000 atmospheres for periods extending from 5 to 30 minutes, the organisms became spheroidal and many underwent cytolysis. The rounding effects were reversible after a recovery period spanning several days. At 2000 atmospheres there was a drastic rounding of the cells, followed by complete cytolysis of all the organisms.

Hodapp and Luyet (1947) studied the mechanism of death of paramecia subjected to high hydrostatic pressure. They obtained a typical sigmoid curve of the percentage of paramecia killed by pressures varied systematically from 500–1200 atmospheres, each pressure being maintained for two minutes. At 950 atmospheres about 50 per cent of the cells were killed. Hodapp and Luyet also varied the time, the temperature, the rate at which the pressure was increased and decreased, and the age of the cultures, and found that the total compression time and the culture age were most important in relation to lethality. Temperature (between 10°–22° C.) and the rate of pressure increase were reported to have little or no effect upon lethality. In this connection, however, it should be realized that Hodapp and Luyet did not employ a windowed pressure chamber and could not observe the effects until after the organisms were removed from the chamber. Consequently, the compression and decompression effects could not be differentiated.

In 1934, D. E. S. Brown, studying frog muscle, first recognized the interrela-

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[1] Work supported by Grant C807 (c 1-10) from the National Cancer Institute, U.S.P.H.S.

tionship between pressure and temperature in biological systems, and an appreciation of this important relationship did much to clarify subsequent investigations in the field. Further insight, particularly with reference to the problem of how pressure induces the solation of protoplasmic gels, was provided by a classification of gelational phenomena, published by Freundlich in 1937. Experiments by Heyman (1935, 1936) had shown that certain gel systems, represented by methyl cellulose, behave oppositely to gelatin. The methyl cellulose type of system displays a volume increment ($+\Delta V$) upon gelation; and Freundlich deduced that such gelations must be endergonic in nature. Then Marsland and Brown (1942) studied the sol-gel equilibria of myosin, methyl cellulose, and gelatin as affected by hydrostatic pressure and temperature. These experiments showed that myosin (*in vitro*), methyl cellulose, and protoplasmic gels, generally, must be placed in a common class of system because all undergo solation as a result of compression and cooling. Gelatin, on the other hand, gelates more firmly with cooling and compression and must represent a different class of system. These workers also emphasized the speed with which protoplasmic sol-gel equilibria may be shifted, particularly in fresh myosin preparations, and they postulated the intervention of an enzyme system (an ATP-ase complex) which likewise is sensitive to pressure and temperature.

The studies of Marsland (1950) demonstrated that the cortical plasmagel of dividing sea urchin eggs reacts to pressure-temperature treatments as do other intracellular gels. Landau *et al.* (1954), performing similar experiments on the plasmagel system of *Amoeba proteus*, came to the same conclusion, namely, that the gel system is weakened by higher pressures and lower temperatures, within the physiological range.

It is apparent, therefore, that pressure-temperature conditions may effect profound changes in cell structure and that such changes are determined, at least partly, by pressure-temperature effects upon the gelational state of the protoplasm. The purpose of the present work, accordingly, is to study the form-stability of two representative ciliates, Blepharisma and Paramecium, under systematically varied conditions of pressure and temperature. Generally, it has been supposed that the characteristic morphology and structural integrity of these organisms is maintained, not only by a tough, flexible surface pellicle, but also by a peripheral gelated layer of cytoplasm, often referred to as "ectoplasm." In the previous pressure studies on Paramecium, the organisms were not observed clearly during the compression period so that compression effects could not be distinguished from the effects of decompression; and in the previous studies on Blepharisma (Hirshfield *et al.*, 1957) no extensive variation in temperature was employed.

MATERIALS AND METHODS

The ciliates, *Blepharisma undulans* and *Paramecium caudatum*, provided excellent material for this study because of their elongate shape which tends to become spheroidal when structural instability develops.

The original Blepharisma culture was obtained from Dr. H. I. Hirshfield; and the paramecia were derived from a mass culture maintained for many years at New York University. Both species were cultured in a lettuce-*Pseudomonas ovalis* medium according to the method of Hirshfield (personal communication). The Blepharisma were maintained in an incubator at a temperature of 20° C., whereas the paramecia were kept at room temperature (25°–27° C.).

Periodically, single organisms were isolated and placed in the separate spots of a 12-spot Klein agglutination slide for five days. The contents of each depression were then transferred to a test-tube of lettuce medium which had been inoculated three days previously with *Pseudomonas ovalis.* Such Blepharisma clones were used for experimental purposes only from the seventh to the eleventh day after isolation. These gave fairly consistent data, but organisms from older cultures displayed a marked increase in pressure sensitivity. Paramecium cultures were more stable and gave fairly consistent results over a three-week period.

The constant-temperature housing, used with the pressure apparatus, has been described by Marsland (1950). The apparatus provides for a rapid build-up and release of pressure, and for constant microscopic observation while the organisms are under pressure. In the pressure bomb the protozoa were kept in view by confining them within a small plastic chamber (6.5 mm. diameter and 2 mm. depth) which was closed above and below by glass coverslips held in position by Lubriseal films.

Ten to thirty ciliates were placed in the chamber for each experiment, and the remaining volume (approximately 85 ml.) of the pressure chamber was filled with Brandwein solution. The duration of exposure to any given pressure was fifteen minutes. The pressures ranged from 7000 to 11,000 psi. and the temperatures employed were 12°, 15°, 20°, and 25° C. The organisms were counted several times during the progress of each experiment, and then at the end of the 15-minute period the percentage of cytolyzed individuals was determined. At least 60 Blepharisma in a total of four or more experiments were used at each pressure and temperature, at least in all critical ranges.

RESULTS

Blepharisma: pressure-temperature effects on form-stability

Blepharisma from old mixed cultures were very sensitive to pressure. In such cultures (at 20° C.) 4000 psi. (lbs./in.^{2}) usually was sufficient to cause a rounding up of all the specimens and subsequently cytolysis occurred in over 75 per cent. Also, aging cloned cultures showed a steady decrease in resistance to pressure. In fact, during the fourth or fifth week after cloning, pressures of about 4000 psi. became sufficient to cause breakdown of the organisms, as was the case with the mixed cultures. Occasionally, young cloned cultures were found which displayed a similar super-sensitivity to the pressure-temperature conditions. Perhaps such clones were derived from a weak or aberrant individual. In any event they were not used for further experimentation.

Blepharisma from typical young cloned cultures showed little or no tendency to become rounded, regardless of the experimental temperature, until the pressure exceeded 7000 psi. Moreover, there was virtually no cytolysis within the 15-minute experimental period. At higher pressures, however, a number of the specimens first became rounded and then cytolyzed. Furthermore, the temperature of the

TABLE I

Percentage of cytolysis in Blepharisma after 15-minute exposure to various pressure-temperature conditions. These are the results of the individual experiments. In each experiment the number of lysed specimens is given in relation to the total number treated

T° C.	psi.			
	8000	9000	10,000	11,000
12	10–20 13–25 8–15 31–60 = 52%	12–15 15–20 27–35 = 77%	17–18 = 100%	—
15	4–20 3–12 9–23 7–15 23–70 = 33%	6–10 7–13 10–20 10–15 8–13 11–17 52–88 = 59%	9–10 23–25 11–12 13–15 20–22 76–84 = 91%	15–15 = 100%
20	5–25 3–15 2–10 3–10 4–13 2–10 3–10 22–93 = 24%	7–15 5–15 6–18 8–20 5–15 4–11 4–11 39–105 = 37%	6–10 9–10 14–15 9–10 13–15 51–60 = 85%	14–15 8–8 15–15 37–38 = 100%
25	3–20 2–26 2–16 2–22 9–82 = 11%	3–10 4–11 4–14 4–10 3–10 6–15 24–70 = 34%	5–6 7–8 17–20 10–12 13–17 9–12 13–16 74–91 = 81%	15–15 = 100%

experiment had a distinct influence upon the percentage of suseptibility, as is shown in Table I.

The character of the rounding and of the subsequent cytolysis varied somewhat in relation to the intensity of the pressure treatment and to the experimental temperature. However, under *critical conditions*—which may be defined as any pressure-temperature combination which yields just 50 per cent cytolysis in 15 minutes—the reactions were generally similar. Thus it is possible to describe the variations which occurred under sub-critical, critical, and super-critical conditions which, respectively, yielded more and more cytolysis within the experimental time.

The rounding and cytolysis reactions under slightly sub-critical conditions (9000 psi./25° C.) are shown in Figure 1. Under such conditions, generally speaking, the shortening seldom exceeded 25 per cent of the original length; the number of rounded specimens increased only gradually during the experimental period; and the tapered anterior end of the organism tended to retain a fairly close semblance of its original architecture. Generally, motility was absent or at least drastically retarded in the rounded specimens.

Lysis, as was the case under all conditions studied, occurred only subsequent to the rounding reaction. Under sub-critical conditions the time of the lysis was distributed quite evenly throughout the test period. In each specimen, however, the lysis was sudden, sometimes being initiated in the tapered anterior end (Fig. 1, C), and sometimes in the swollen posterior half, near the contractile vacuole. It appeared to involve a sudden rupturing of the cell surface and a disruption of the cytoplasm into a number of rounded free-floating pieces (Fig. 1, D). Occasionally, some of these protoplasmic fragments become motile after pressure was released.

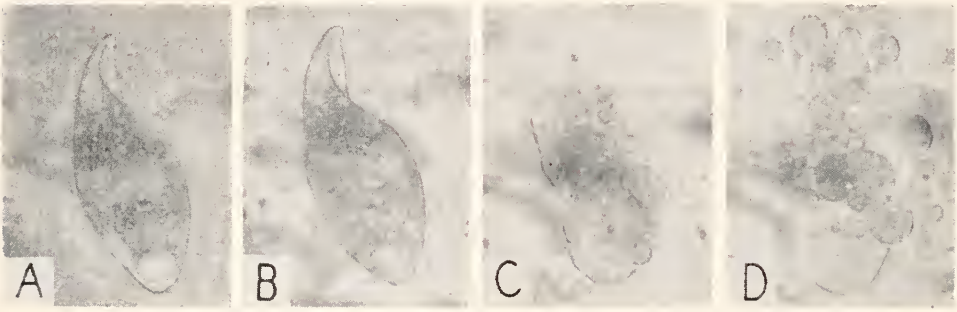

FIGURE 1. Blepharisma: rounding and cytolysis reactions under sub-critical conditions (9000 psi./25° C.). A and B: Gradual shortening of specimen; successive exposures taken 4 and 7 minutes after pressure build-up. C: Sudden cytolysis, exposure 30 seconds after B. D: Rounding up of cytoplasmic remnants, one minute after cytolysis. After decompression some of these fragments may become motile. Photographs retouched.

Under distinctly super-critical conditions, rounding and lysis developed rapidly and most of the susceptible specimens had reacted within the first five minutes. The shortening of specimens was distinctly greater, although often there was some persistence of the tapered anterior end up to the moment of lysis. The lysis was more complete; the protoplasmic fragments were smaller; these fragments showed less tendency to round up, and they did not develop motility subsequent to decompression.

Under intermediate conditions, in and around the critical range, the rounding and cytolysis reactions were intermediate in character.

Blepharisma: decompression effects

The sudden release of pressure, under critical or nearly critical conditions, gave rise, within two minutes, to an abrupt, further shortening of all the non-cytolyzed specimens, accompanied by a momentary stoppage of any persisting ciliary activity.

This shortening (Fig. 2) was more abrupt than the pressure-induced rounding. Immediately after shortening, a few specimens displayed sudden lysis, but this *decompression lysis* did not involve more than five per cent of the animals. In fact, most of the specimens regained their motility within some ten minutes, and after 30 to 200 minutes they presented a fairly normal form and appearance.

Blepharisma: pressure-temperature parameters of cytolysis

As may be seen in Table I, the percentage of cytolysis obtained at any given pressure represents a temperature-dependent value. The sensitivity to pressure cytolysis increases very definitely with decreasing temperature within the experimental range (25°–12° C.). This is shown more clearly when the data are plotted, as in Figure 3. Conversely, the resistance to pressure cytolysis increases with increasing temperature, as is shown in Figure 4. There it may be seen that the pressure which is just adequate to induce cytolysis in 50 per cent of the treated specimens increases regularly as the temperature increases, within the given range.

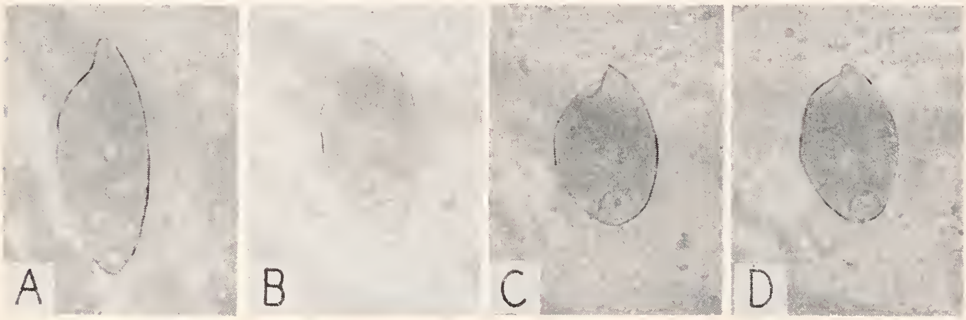

FIGURE 2. Blepharisma: shortening under pressure (slightly sub-critical conditions, *i.e.*, 9000 psi./25° C.) followed by rapid shortening after decompression. A: Exposure 13 minutes after pressure build-up. B: One minute after decompression. C: Two minutes later. D: Another two minutes later. Photographs retouched.

Blepharisma: pressure-centrifuge experiments

Quantitative measurements of the solational effects of pressure are difficult to obtain with Blepharisma. The pressure-centrifuge method, which has been used widely for other cells (Marsland, 1956), is not very suitable. The orientation of the specimens in the centrifugal field shows considerable variation, and the instability of form and cellular integrity at higher pressures gives further difficulty.

It was possible, however, to obtain qualitative data which showed unequivocally that pressure does induce solational changes in the cytoplasm of Blepharisma. Many of the specimens centrifuged for one minute at 5000 × gravity at 3000 psi. showed a distinct clearing of the centripetal half of the cell—by virtue of the centrifugal displacement of food vacuoles and other granular bodies—to a degree that was never found in control specimens, centrifuged simultaneously at atmospheric pressure.

Paramecium: comparative observations

Generally speaking, the pressure-temperature effects on Paramecium and Blepharisma were similar. However, there were two important differences: 1) Paramecium was distinctly more sensitive to pressure lysis, and 2) *decompression lysis,* which was almost negligible in Blepharisma, became very significant in Paramecium.

For Paramecium, the critical pressure for 50 per cent lysis was 2000–3000 psi. lower than for Blepharisma, at each of the two temperatures (20° and 25° C.) which were studied. Under such critical conditions (*e.g.*, 7000 psi./20° C.) the animals shortened moderately and displayed gradually diminishing, distinctly irregular locomotion, which ceased only if and when cytolysis occurred. Most of the cytolysis occurred during the last 5 minutes of the 15-minute compression period. Moreover, two somewhat different types of lysis were observed with roughly equal frequency. One type seemed to involve a detachment of the pellicle, with the formation of one or more large hyaline blisters which later broke, liberating the deeper granular cytoplasm (Fig. 5). The other type, in contrast, seemed to

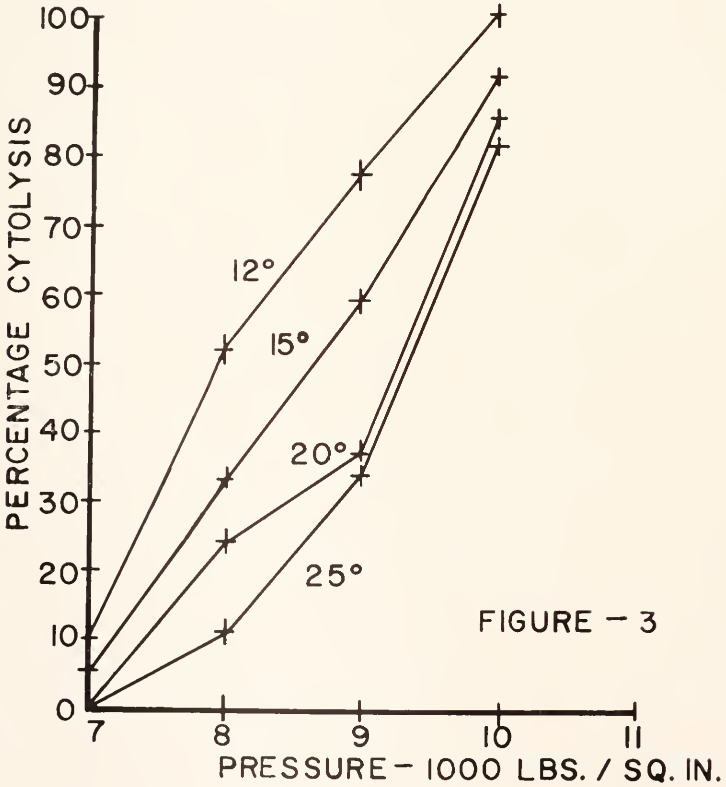

FIGURE 3. Blepharisma: percentage of cytolysis as a function of pressure, at four temperatures. Cytolyzed cells were counted exactly 15 minutes subsequent to pressure build-up.

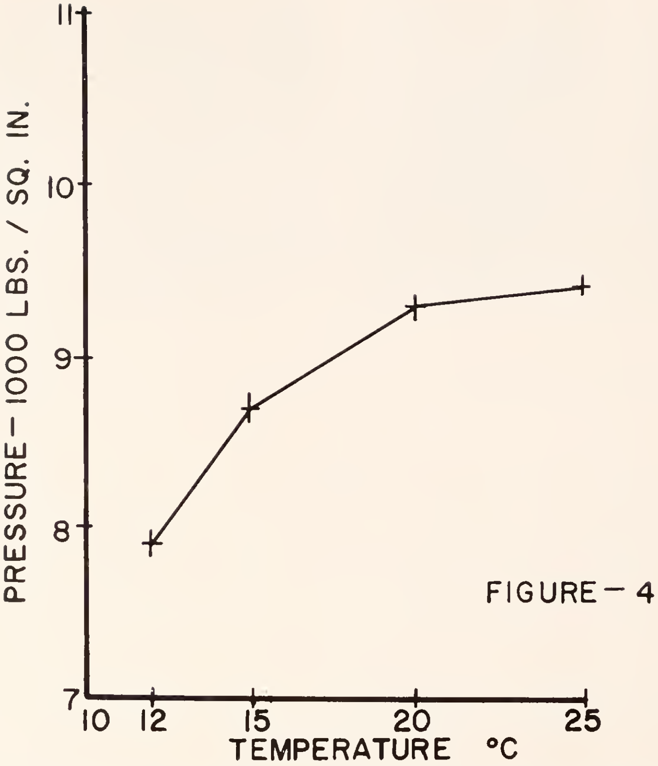

FIGURE 4. Blepharisma: critical pressure yielding 50 per cent cytolysis after 15-minute exposure, plotted as a function of temperature. The data of this figure are derived from Figure 3.

represent a more generalized breakdown of the cell surface in either the anterior or posterior half of the animal, with a less abrupt scattering of the granular cytoplasm (Fig. 6). With Paramecium, moreover, regardless of the conditions or type of cytolysis, there was very little tendency for the cytoplasmic remnants to round up, or to wall themselves off from the surrounding medium.

The decompression lysis under critical conditions usually involved more than half of the surviving specimens, particularly when the decompression was rapid (within one second). Sudden decompression was followed within about two minutes by an abrupt further shortening of all surviving specimens, followed immediately by a generalized cytolysis of the majority. Specimens that escaped cytolysis, on the other hand, gradually regained normal form and motility within 2–3 hours.

Under super-critical compression (8000 psi./20° C.), the degree of rounding was greater; and most of the lysis, which involved more than 60 per cent of the specimens, occurred during the first ten minutes of the compression period. Then, following rapid decompression, *all* surviving specimens shortened still more and quickly underwent cytolysis.

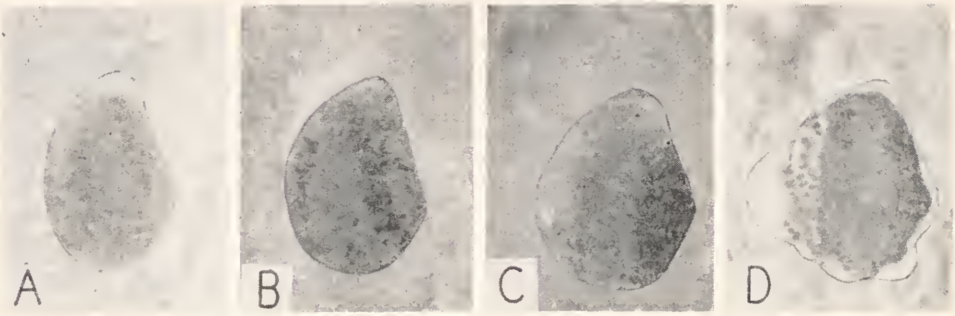

FIGURE 5. Paramecium: one type of pressure cytolysis (8000 psi./25° C.). A and B: Shortened specimen, photographed 9 and 10 minutes after compression. C: Two minutes later, cytolysis starting. D: 30 seconds later, showing hyaline blisters which are about to break. Photographs retouched.

Distinctly sub-critical conditions (5000 psi./20° C.) produced no clearly defined effects; but at 6000 psi. a slight degree of shortening was noted, although locomotion appeared to continue in normal manner. Abrupt decompression, under these conditions, produced a further sudden shortening of the specimens, but there was no cytolysis and usually the animals regained their normal form within one hour.

DISCUSSION

The problem of how pressure exerts its effects upon cellular systems has been approached from several angles. Regnard (1891) interpreted his results in terms of an imbibition of water by the ciliated cells. However, since no volume increase can be found in pressurized cells, this hypothesis has not been pursued. Hodapp and Luyet (1947) suggested a disturbance of the permeability mechanism and an injury of the neuromotor apparatus as the main factors involved in pressure lysis. However, as stated previously, they were unable to observe the organisms during the pressure period. As to their findings that temperature and the rate of application and release of pressure had no effect on lethality, at the high pressures they employed, it is probable that the decompression effects were very drastic and negated these variables.

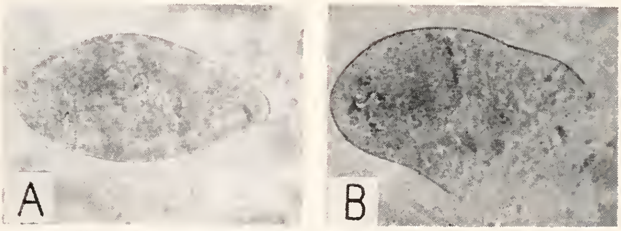

FIGURE 6. Paramecium: another more generalized type of pressure lysis (9000 psi./25° C.). A: Shortened intact specimen 5 minutes after pressure build-up. B: 5 minutes later, sudden cytolysis initially involving all of the anterior half of the specimen. Photographs retouched.

Pressure-temperature effects on cell form

A more fruitful approach, perhaps, is to interpret the observed effects on the cell form and integrity of Blepharisma and Paramecium in terms of the now well established action of pressure and temperature upon intracellular gel structures. To some extent this approach has been adopted by Ebbecke (1936), who had, however, very little experimental evidence. Moreover, Ebbecke postulated that pressure exerts its effect upon the gel system indirectly via an action upon cell metabolism, rather than directly *and* indirectly, as proposed by Marsland and Brown (1942).

It now seems reasonable to assume that the cortical cytoplasm of ciliates, immediately subjacent to the pellicle, is firmly gelated and that this plasmagel layer plays a significant role in helping to maintain the unique form of the particular species. Also, it seems possible that the plasmagel layer of the ciliate may possess contractile properties which can be instrumental in producing changes of form and orientation during normal locomotion.

Experimental evidence in regard to the foregoing question is not very extensive, however. A plasmagel structure is indicated, to be sure, by the fact that the peripheral layer of cytoplasm, in which the trichocysts are lodged, does not become involved in the protoplasmic streaming when cyclosis occurs. Also, it is frequently observed, not only in the pressure-temperature experiments, but also when Paramecium and other ciliates are exposed to toxic substances or merely flattened under a coverslip, that the pellicle may peel away from the subjacent cytoplasm and form a hyaline blister of large or smaller size. When this happens the granular cytoplasm, from which the pellicle has become detached, may persist, retaining its stability for a minute or two. Then it disintegrates and pours forth its granular components into the hyaline fluid which fills the blister.

All the evidence of the present experiments indicates that the shortening and rounding of the cells induced by suitably high pressure and modified by temperature are mediated by a solation of the plasmagel layer. Qualitatively, the susceptibility of this gel to pressure solation is established by the pressure-centrifuge experiments and quantitatively the pressure-temperature parameters of this gel system are very similar to those which have been established in various other protoplasmic gels (Marsland, 1956). Apparently a shortening and rounding of these elongate ciliated cells occur, under the agency of tensional forces in the cell surface, whenever the subjacent plasmagel structure is weakened below a certain critical resistance level.

Pressure-temperature lysis

Pressure lysis, apparently, is always preceded by a rounding of the cells; and, generally speaking, the more drastic the rounding the greater is the lysis tendency. It seems likely, therefore, that cell form and cell integrity may be determined by similar underlying factors.

A firmly maintained plasmagel structure would serve, most probably, not only to stabilize the total form of the cell, but also to preserve the orientation and spatial configuration of many of the microscopic and submicroscopic constituents of cell structure. Moreover, if the solation is drastic enough to allow for a rounding of the cell, the rounding itself tends to disturb and disorient the configuration of the protoplasmic constituents. Cytolysis, perhaps, may involve a detachment of the pellicle from the subjacent plasmagel, with a concomitant disarrangement of the ciliary origins and trichocysts, or it may involve some other type of disorientation. In any event, it seems to occur whenever drastic solation occurs. Thus it is not surprising to note that preliminary dosages of UV-irradiation, utilizing wave-lengths which have a primary effect upon the proteins of the peripheral cytoplasm, predispose Blepharisma to pressure cytolysis, presumably as a result of a weakening effect upon the plasmagel structure (Hirshfield *et al.,* 1957).

Decompression lysis

This phenomenon, which was particularly conspicuous in Paramecium, can be interpreted, perhaps, in terms of a rapid post-pressure reconstruction and contraction of the plasmagel system. A similar phenomenon, in fact, has been described for Amoeba by Landau, Zimmerman and Marsland (1954). Many studies have shown that pressure solation is rapidly reversible upon decompression and the Amoeba study indicates that the newly reconstituted gel system tends to contract sharply, presumably as a result of an accumulation of metabolites which are not fully utilized during the pressure period (Landau *et al.,* 1954). In any event, both Blepharisma and Paramecium always showed an abrupt contraction about two minutes after release from any extensive critical or super-critical pressure-temperature treatment and, particularly in the case of Paramecium, this abrupt contraction was very frequently accompanied by cytolysis. Precisely why cytolysis should occur under these circumstances is problematical. It may be supposed, however, that such a contraction would tend to disrupt the surface architecture of the cell, especially if it occurs before a proper stabilization of the cell structure has occurred. Furthermore, these observations indicate that the peripheral gelated cytoplasm of the ciliate displays a potential contractility, and that this layer may play a role in effecting changes of form and orientation, during locomotion, and in performing the work of cell division.

Metabolic relationships

The increasing susceptibility of older cloned cultures in regard to pressure-temperature cytolysis raises some interesting questions. A continued source of metabolic energy appears to be necessary for the maintenance of protoplasmic gel structures (see Marsland, 1956); but why should such structures tend to be weaker in aging clones? Lettré (1952) has suggested that cell form and stability may be dependent upon the level of ATP reserve in the cell, but why should this tend to diminish with age? ATP-sensitive proteins, capable of forming potentially contractile gel systems, seem to be present in various relatively unspecialized cells—in the slime mold (Loewy, 1952 and Ts'o *et al.,* 1956), in sea urchin eggs (Mirsky, 1936), in fibroblasts and other tissue cells (Weber, 1955 and Hoffman-Berling, 1954), and in Amoeba (Landau *et al.,* 1954). At present, however, it is entirely problematical as to whether age-changes in the gel structure result from changes in metabolism, changes in the constituent proteins or, at least partly, from other unknown changes.

SUMMARY

1. Two ciliates, *Blepharisma undulans* and *Paramecium caudatum,* were studied with reference to form stability and integrity (resistance to cytolysis) under varying conditions of hydrostatic pressure (up to 10,000 lbs./in.^{2}) and of temperature (12°–25° C.).

2. At lower pressures the specimens retained their elongate form, but at higher levels, depending on temperature, species, and age of the cloned cultures, the cells gradually become shorter and more rounded. Following this form change, cytolysis occurred in a varying percentage of the specimens. Older cloned cultures showed a greater and more variable susceptibility to the pressure-temperature effects, so that selected younger cultures were used for the quantitative evaluations.

3. For Blepharisma, the critical pressure, which gave 50 per cent cytolysis within a 15-minute compression period, displayed a distinct temperature dependence, being 8000 psi. at 12° C., 8700 at 15° C., 9200 at 20° C., and 9300 at 25° C. Paramecium, in contrast, showed a distinctly greater sensitivity, the critical pressure for 50 per cent cytolysis at 20° C. being some 2000 psi. lower than for Blepharisma.

4. Rapid decompression, following any critical or super-critical pressure treatment, produced an abrupt further shortening (contraction) of the specimens, accompanied by a cytolysis of some of the previously resistant individuals. For Blepharisma, decompression cytolysis involved only about 5 per cent of the animals. Paramecium, however, was much more sensitive and virtually 100 per cent became involved.

5. An interpretation of these changes in cell form and integrity is given in terms of pressure-temperature effects upon protoplasmic gel structure, particularly with reference to the solation of the peripheral plasmagel layer of the cytoplasm.

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OBSERVATIONS ON THE SYMBIOSIS OF THE SEA ANEMONE STOICHACTIS AND THE POMACENTRID FISH, AMPHIPRION PERCULA[1]

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The partnership between certain tropical damselfishes and sea anemones has excited the interest of students of natural history for almost a century. The most significant investigations of the symbiosis have been those of Sluiter (1888), Verwey (1930) and Gohar (1948), who have given us some knowledge of the ecology and behavioral characteristics of the animals. In 1947 Gudger reviewed all the observations that had been made up to that time, and in 1950 Baerends first speculated about the possible role of releasers in the maintenance of the association.

However, this symbiosis, like many others, still poses many unanswered questions. The physiological and behavioral mechanisms which maintain the animals in partnership have not been investigated with present-day techniques.

It has not been clear whether the fish responds to chemical, tactile or visual stimuli from the host, nor whether the behavior of the anemone is affected by stimuli from the fish. The mechanism whereby the fish is protected from the nematocysts of the host has been a mystery. In spite of the fact that it is generally supposed that nematocysts are not under nervous control but that they fire off independently upon adequate stimulation, several investigators have speculated that in such partnerships the presence of the fish in some way causes the coelenterate host to put its nematocysts "out of action" (Baerends, 1957, p. 262). The question remains whether the fish simply fails to provide adequate stimuli to discharge the nematocysts, or whether a factor is produced by the fish which markedly raises the threshold of discharge of the nematocysts and thus affords protection. Finally, it remains to be determined whether or not the fish is immune to the poison of the nematocysts.

Recently at Marineland of the Pacific it became possible to investigate the partnership between *Amphiprion percula* (Lacépède) and the giant anemone *Stoichactis* (Fig. 1). We directed our attention primarily to the physiological and behavioral mechanisms involved in the protection of the fish against the nematocysts of its host and in the course of the work were able to re-examine and re-establish some of the observations of Verwey and Gohar.

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[1] Contribution No. 6, Marineland of the Pacific Biological Laboratory. This work was carried out under the contract of the senior author with the Office of Naval Research. We wish to express our appreciation to Marineland of the Pacific for its hospitality and facilities and to Dr. Cadet Hand for a tentative identification of our anemone.

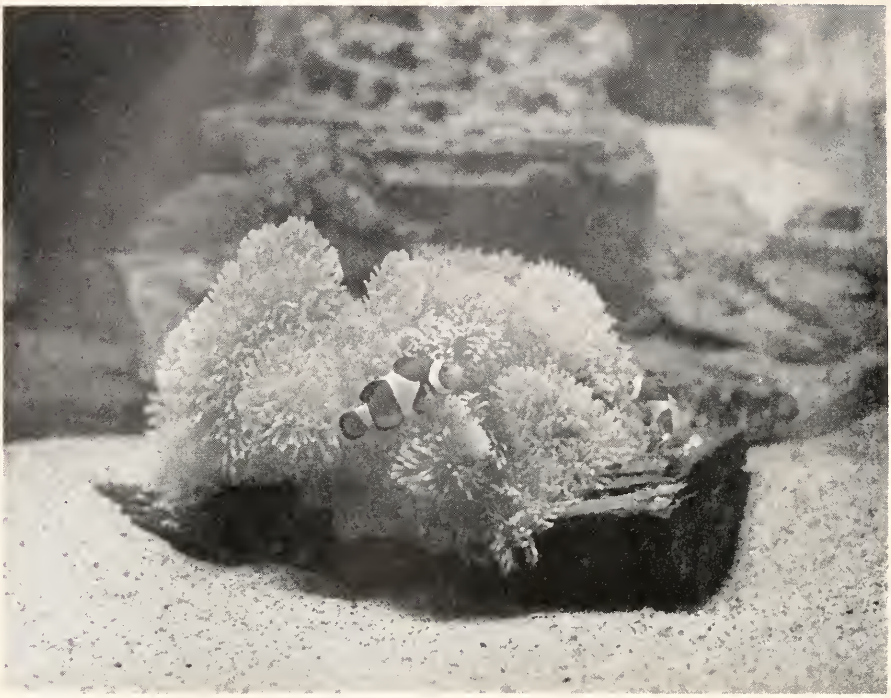

FIGURE 1. The anemone Stoichactis and two partner *Amphiprion percula*. Photographed in the exhibition aquarium at Marineland of the Pacific. Approximately × 1/6.

MATERIAL AND METHODS

Experimental fish were obtained on the reefs near Nasugbu, Batangas Province, Luzon, in the Philippine Islands by commercial collectors. Our single specimen of the host anemone was taken at the same locality. Verwey (1930) describes an anemone, probably identical with ours, from Batavia Bay, Java (Anemone 1, Plate XV, Fig. 2) which he says is colonized in nature by *Amphiprion percula* alone.[2] Ours was provided by the collectors specifically as the host of *A. percula.* At this writing it is still alive at Marineland. We believe it to be *Stoichactis kenti* (Haddon and Shackleton) although precise identification will not be possible until examination of the internal anatomy can be made after the animal is preserved.

The anemone was received at Marineland in January of 1957, and our experiments were started on September 3, 1957. Thus the animal was acclimated to Marineland sea water for a period of somewhat over 8 months. During the period of our observations it was maintained at 25° C. in a 60-gallon "photographic" redwood aquarium, which was so constructed that a sheet of glass could be inserted to isolate fishes from the anemone when desired.

We received a total of thirteen specimens of *Amphiprion percula.* The previous history of these fish is totally unknown to us. Probably some or all of the animals were collected from anemones. However, according to Dr. José Montilla of the Division of Marine Fisheries in Manila, this species of Amphiprion does not always live in association with anemones in the Philippines; hence some of our experimental fish may have been free-living. Also, it is known that *A. percula* lives in association with at least two species of anemones (Verwey, 1930). Therefore the host habit of any that may have been commensal is also unknown to us.

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[2] In the aquarium at Batavia this anemone was readily occupied by *Amphiprion akallopisus* and *A. polynemus,* in addition to *A. percula.*

Prior to the experiments, two fish, A and B, were kept in partnership with the anemone for several weeks. These gave us controls which we knew were "acclimated" to the anemone. Nine other fish (C to K) had been isolated from any possible sensory contact with an anemone host for a period of not less than six weeks. In the following experiments these are spoken of as "unacclimated" fish.

Two other *A. percula* (L and M) which had occasionally been put in with the anemone for exhibition purposes prior to our experiments were also used. One of these (L) was the largest animal in our sample, measuring 65 mm. standard length. Fish M, a small animal, was sacrificed in a physiological experiment.

The age and sex of our *Amphiprion percula* were not determined.

A single adult specimen (56 mm. standard length) of *Amphiprion frenatus* Brevoort was available for specificity studies. This fish had lived in the exhibition aquarium with the anemone for the eight months prior to our experiments but was never observed to enter it.

The most careful precautions were taken to maintain all glassware, forceps, scissors, dip nets, and other tools free from contamination with organic materials, because of the well-known sensitivity of nematocysts to such substances. All items employed in the manipulation of fishes or isolated anemone tentacles were scrubbed with detergent, washed in distilled water, dipped in ether-alcohol and allowed to dry without contact. Fish to be sacrificed were dissected with clean instruments in clean Petri dishes. Whenever possible, experimental fish were not handled at all but were trapped in the aquaria with clean 500-cc. beakers. When it was necessary to use a nylon dip net, the net was first boiled and rinsed.

Experiments on the discharge of nematocysts from isolated tentacles were conducted in clean watch glasses. A new tentacle was prepared for each test. Tentacles were isolated by clipping them off at the base with clean, fine-tipped scissors. They were stimulated mechanically with a clean glass rod drawn to a fine point, and electrically stimulated with a platinum wire-glass electrode drawn to a fine capillary point. The electrode was connected in a circuit with a standard inductorium, key, and a 6-volt dry cell. A small piece of aluminum foil dipped into the sea water in the watch glass served as the other electrode. Between each experiment, the watch glass, the glass rods, the platinum wire, and the capillary tube were washed with ether-alcohol; the capillary tube was refilled with clean sea water, and the aluminum electrode was replaced.

In certain experiments, one-cm. cubes of plastic sponge were used. These were cut from the center of a new commercial sponge by use of a clean single-edge razor blade.

Observations on the "Process of Acclimation"

It has been observed (Gohar, 1948) that the acclimation of an Amphiprion to an anemone may take a considerable length of time. The details of this acclimation remain virtually unknown. We felt that careful observation of this process might give us insight into the mechanism which protects the fish from nematocyst discharge.

Accordingly, a series of nine experiments were performed in which we introduced individual unacclimated *Amphiprion percula* into the observation tank with the anemone. These tests revealed a fairly stereotyped series of events which terminated in the acclimation of each new fish to the anemone. The results of these experiments are summarized below.

An unacclimated fish introduced into the tank a foot or so away from the anemone, usually approached the anemone within a few minutes and began to swim under the disk, around the column, and occasionally over the top of the disk a centimeter or more away from the tentacles. Such fish spent most of their time under the disk at this stage and sometimes were seen nibbling at the column of the anemone. Most fish seemed to "recognize" the anemone within a few minutes and swam toward it. However, in two tests, two fish failed to react noticeably to the anemone for 20 and 27 minutes, respectively. In both cases another fish was introduced directly onto the disk of the anemone where it shortly took up residence. In both tests the unreactive fish then came rather quickly toward the anemone, apparently in response to the fish already in occupancy, and began the characteristic acclimation process.

As the process proceeded, passage over the disk became more and more frequent and the "acclimating" fish moved closer and closer to the tentacles. Swimming was accomplished by a distinctive series of slow vertical undulations, in which the tail was usually held a little lower than the rest of the body. Eventually, on one of these trips over the disk, the fish would touch a tentacle or two, usually with the ventral edge of its anal fin or the lower margin of its caudal fin. Commonly this resulted in a moderate adherence of the tentacle to the fin and contraction of the tentacle. The fish then jerked itself free with a violent flexure of its body and usually raced off the disk. Not all newly introduced Amphiprion caused clinging upon their first contact with tentacles, but it was the general rule. However, this adherence failed to deter the fish, which nearly always returned immediately to the anemone, either under the disk or over the tentacles. In our experiments the time from initial introduction until the first physical contact between fish and anemone varied from less than 1 minute to 65 minutes.

After this initial contact the fish typically came closer and closer to the tentacles, touching them with increasing regularity. The reaction to the clinging of tentacles became less and less violent until a sudden flexure of the animal's body was the only reaction given by the fish. Mouthing or nipping of tentacles was often observed in this and later stages.

The clinging and contraction of tentacles upon contact with the fish gradually became less until it ceased altogether. At the same time the fish began to swim deeper among the tentacles, using the same slow undulating movements as when it had cruised above the disk.

Once the fish was swimming in fairly constant contact with the tentacles of the anemone, a very striking change in its behavior occurred. The general speed of swimming suddenly increased until the Amphiprion was dashing back and forth over the disk of the anemone, flailing unreactive tentacles aside with violent movements of its body. Often the fish raced beneath the anemone and appeared in one of the folds of the disk margin, its head completely ringed in tentacles. The fish frequently maintained this vantage point for a few seconds, holding position with rapid alternate fanning movements of its pectoral fins, after which it might dash onto the disk again for another foray among the tentacles. The powerful swimming typical of this stage of the acclimation process was accomplished by rapid and strong lateral body flexures. The impression given by the swimming behavior of the fish after final acclimation was that the fish was "bathing" its entire skin surface among the tentacles.

At this point we considered the fish to be fully acclimated to the anemone, since no further clinging or tentacle contraction appeared. The time required for complete acclimation varied from about one minute to nearly three hours, with an average time of one hour.

If a fully acclimated fish was removed from the anemone and its fins or body carefully scraped with a scalpel, and then returned to the anemone, the scraped areas caused both clinging and tentacle contraction. However, fish treated in this manner did not then begin the acclimation process anew but stayed among the tentacles until clinging waned and disappeared. These fish gave evidence of discomfort from the clinging tentacles by jerking themselves free. They did not, however, rush off the disk. It would seem that treating the fish in this way partially broke down their protection.

Acclimation involves development of visual recognition of the anemone by the fish. This was demonstrated by removing fully acclimated fish from the anemone and placing them in a compartment of the observation tank separated from the anemone by a heavy glass sheet. Incoming water was introduced into the isolation compartment, flowed over and around the partition, and was discharged from the compartment containing the anemone to prevent chemical gradients from occurring which could guide the fish. In every case acclimated fish oriented strongly toward the anemone which they could see through the glass, by gathering at the glass nearest it and swimming up and down with their heads directed toward their host.

The behavior of an Amphiprion which has been resident for a time in an anemone is somewhat different from that of a newly acclimated animal. The general level of activity becomes lower though such a fish normally moves much more rapidly than an unacclimated fish. After acclimation of the fish is complete the anemone tends to become a strongly defended territory. Acclimated fishes often refuse to leave the anemone's folds even if it is lifted from the water.

Experiments on Protection Against the Host

In these experiments we wished to determine initially whether the presence of the fish close to but not in contact with the surface of the anemone had any *observable* effect on the anemone.

Experiment No. 1. A ½-inch I.D. plastic tube was cleaned with alcohol-ether. A small *A. percula* was slipped into the tube and shaken down it until it protruded slightly from the end. When the fish was held as close as ½ mm. from the tentacles, they showed no reaction whatever. A similar test with a control *Fundulus parvipinnis* gave identical results. Contact of a single tentacle with the Fundulus resulted in immediate massive discharge and clinging.

No interaction at a distance between the partners or between prey and anemone could be observed.

Next, in the hope that we might be able to identify and localize the mechanism of protection, we designed the following experiments in which direct stimulation of the anemone was employed.

Experiment No. 2. As a control, we investigated the reaction of the anemone to stimulation with a clean, flame-polished glass rod. In a number of repeated tests we saw that such stimulation caused "clumping" of the tentacles, marked adherence to the rod (discharge of nematocysts), retraction of the tentacles, and retraction of the lobe of the disk in the vicinity of the point of stimulation. Far greater mechanical stimulation and agitation of tentacles and disk by Amphiprion produce no noticeable response from the anemone.

Experiment No. 3. We trapped an Amphiprion in a beaker and held it by the lower jaw in the tips of a pair of fine-tipped forceps. Twice we drew it forcibly across the disk of the anemone, bringing it into violent contact with the tentacles. There was no discernible reaction from the anemone. The fish when released immediately entered the tentacles in a normal manner and "bathed" itself among them.

An adult *Fundulus parvipinnis* was brought into contact with the anemone and was seized in the characteristic way, involving widespread adhesion, tentacle contraction, and infolding of the disk.

An *Amphiprion percula*, trapped in a beaker and held with forceps by the jaw, was brought into contact with a large specimen of the eastern Pacific anemone, *Anthopleura xanthogrammica*. There was immediate widespread clinging so that the fish had to be pulled forcibly from the anemone.

Experiment No. 4. An Amphiprion was sacrificed, and we cut a cross-sectional piece of flesh, including skin, from it with a carefully cleaned scalpel. We made a similar preparation from Fundulus. The two preparations were placed next to each other on the disk of the anemone. The flesh from Amphiprion was slowly worked to the edge of the disk and cast off, while the Fundulus meat was enveloped and ingested. The experiment was later repeated with similar results.

Experiment No. 5. We caught an Amphiprion, placed it in a clean Petri dish and killed it by severing the head. We then dissected off a strip of skin, taking the greatest care to prevent contact of both surfaces of the strip with other skin surfaces. We brought this piece of freshly-removed skin into contact, *on its outer surface*, with several tentacles of the anemone. No clinging occurred except for slight adherence at the edge of the piece of skin. When the skin was brought into contact *on its inner surface*, the tentacles immediately clung strongly to it.

This experiment was repeated twice with identical results. Strips of skin from the same fish were used.

Experiment No. 6. We heated the two pieces of skin used in the preceding experiment to 90° C. for ten minutes in sea water in separate clean test tubes. The preparations were cooled. When we brought the outside surface of these heat-treated pieces into contact with tentacles, clinging immediately occurred.

Experiment No. 7. A ½-cm. cube of muscle without skin was cut from the caudal peduncle of the *Amphiprion percula*, taking great care not to bring it in contact with skin surface. It was placed on the disk of the Stoichactis, and was immediately seized. The tentacles clumped around the piece and infolding of the disk margin occurred. The anemone's response differed in no discernible way from its response to Fundulus meat.

Experiment No. 8. Four cubes were cut from a commercial plastic sponge.

In the following tests, the "clinging reaction" of a small group of tentacles was tested. When the plastic cube was brought in contact with the tentacles, the reaction was classified arbitrarily from 0 (no clinging) to ++++ (very strong adhesion). In each test a different group of tentacles was selected. The time required for release of the cube was noted.

a. A clean control cube: tentacles retracted; clinging 0-+; time of release < 1 second. This control was repeated several times with identical results.
b. A similar cube of which all surfaces had been rubbed over the skin of *Amphiprion percula:* results identical with the control. This test was repeated several times with similar results.
c. A cube rubbed over the skin of *Amphiprion frenatus:* clinging ++; retraction of tentacles, release time 20 seconds.
d. A cube rubbed over the skin of an adult Garibaldi, *Hypsypops rubicunda* (an eastern Pacific *pomacentrid* fish): clinging ++++, released after 2 minutes 45 seconds.
e. A cube rubbed over the skin of *Fundulus parvipinnis:* clinging ++++, released after 3 minutes 45 seconds.

Experiment No. 9. Four new cubes were cut. Two of these were rubbed over *Amphiprion percula.* One clean cube and one mucus-covered cube were heated to 100° C. for ten minutes in a dry oven and cooled.

a. The clean control cube: tentacles retracted; clinging 0-+, time of release < 1 second. Heavy pressure caused sufficient clinging to hold the cube for as long as 4 seconds.
b. A clean cube, heat treated: results identical with control.
c. A mucus-covered, unheated cube: tentacular retraction; clinging 0, even under strong pressure; time of release, immediate.
d. A mucus-covered, heat-treated cube: identical with control (a).

Identical results were obtained in a second series of tests. In this experiment we see that stronger mechanical stimulation than was used in Experiment 8 induced clinging of brief duration in a control sponge. If there was a coating of *Amphiprion percula* mucus on the sponge, clinging could not be induced even with strong pressure. But if the coating of Amphiprion mucus was heat-treated, its protective effect was obliterated.

The effect of heat was also shown in Experiment 6.

Experiment No. 10. On May 14, 1958, while the anemone was located in a display tank, two large groups of eggs were found attached to the rock occupied by the anemone. One patch was being guarded by an adult goby *Bathygobius soporator* (Cuvier and Valenciennes) and the other patch, which was attached in a crevice directly beneath the anemone, was guarded by two adult *Amphiprion percula,* which had been allowed to become resident in the anemone. The eggs of both species were tested for protection against the nematocysts of the anemone.

When the intact egg of the goby was touched against a tentacle, clinging occurred, and the tentacle bent into a clump with four or five other tentacles. No movements of the disk were noted. When eggs were released in the water over the anemone and allowed to drift onto the tentacles the same effects were produced.

When these tests were repeated using Amphiprion eggs, the following results were obtained. Even when an egg was pressed against a tentacle with sufficient pressure to bend the tentacle no clinging resulted. Eggs dropped onto the disk through the water caused no reaction. Quite evidently Amphiprion eggs are as effectively protected as the adult.

Experiments on the Discharge of Nematocysts from Isolated Tentacles

Our next experiments were designed to determine whether Amphiprion mucus raised the threshold of nematocyst discharge. Isolated tentacles were stimulated mechanically or electrically while being observed through a dissecting microscope.

Mechanical stimulation. Pantin (1942) showed that direct mechanical stimulation of the isolated tentacle of *Anemonia sulcata* with a clean glass bead failed to cause discharge. Experiment No. 2 showed that stimulation of the *in situ* tentacle tip of Stoichactis with a smooth flame-polished glass rod results in clinging. Isolated tentacles of Stoichactis appear to be more sensitive to mechanical discharge than those of Anemonia. Even when the greatest care was taken in transferring a tentacle to a clean watch glass in clean sea water for isolation and stimulation, its tip very frequently stuck to the bottom of the glass for a few seconds.

In our preparations mechanical stimulation was effected by fine glass rods or by using the tip of the capillary tube of the glass electrode. Variation in the sensitivity of tentacles, the ease with which nematocysts could be mechanically discharged and our inability to deliver mechanical stimuli of precisely controlled intensity made it difficult to obtain a truly quantitative picture of threshold changes and intensity of discharge.

Our observations on the results of mechanical stimulation by the capillary tube of the electrode may be summarized as follows (discharge classified arbitrarily from 0 to ++++):

a. *Stimulation with the clean capillary tube:* An initial stimulation (light touching) at the tip of the tentacle typically produced a moderate discharge (++). Similar stimulation halfway between the tip of the tentacle and its cut base results in a lighter discharge (+). Repeated mechanical stimulation at both points results in progressively less discharge. Reduction of the discharge is not due to exhaustion of the nematocyst supply, as subsequent electrical stimulation produces massive discharge at the same points.

b. *Stimulation with the tip of the capillary tube covered with a pad of Fundulus mucus (control):* Initial light mechanical stimulation at the tip produced massive discharge (++++) and clinging to the mucus pad.

c. *Stimulation with the tip of the capillary tube covered with mucus from Amphiprion percula:* Light stimulation of both tip and middle of the tentacle produced no discharge (0). If the tentacle was held in place by a clean glass rod and stimulated at another point by the mucus-covered capillary tube so forcibly as to deform the tentacle, the discharge of a few isolated nematocysts occurred but *no clinging resulted.* The effect of the pad of Amphiprion mucus appeared to be limited to the area in contact with the mucus, for if an uncovered portion of the glass tube came in contact with the tentacle, discharge would occur at this point but not at points protected by the mucus pad.

d. *Stimulation with the capillary tube covered with a pad of mucus from Amphiprion frenatus:* Light touch at the tentacle tip → ++. A touch at the side of the tentacle → 0. This test did not appear to be significantly different in results from control (a).

e. *Stimulation with the capillary tube covered with mucus from the base of Stoichactis:* Not significantly different from control (a).

Electrical stimulation. Stimulation by faradic current was produced according to the standard method described above. The single excised tentacle, in clean sea water, was first tested for mechanically-induced discharge by light contact with the electrode at a point halfway between its tip and base. The tentacle was then given a series of three-second bursts of faradic stimulation at the same point, starting with the inductorium at its lowest setting (12).

Table I shows the threshold and intensity of discharge under different conditions in a series of tentacle preparations. Intensity of discharge was arbitrarily classified from 0 to ++++. The sensitivity of the control series in which mucus was absent varied widely. It appears that this variation in sensitivity reflects variation in the threshold of the different preparations, since after the initial mechanically-induced discharge, stimulation by pressing the electrode against the side of the tentacle elicited no further discharge in the four preparations. When a pad of Fundulus mucus was placed over the tip of the electrode, the very lightest mechanical contact of the electrode tip with the side of the tentacle elicited some discharge (+). Hence, if the threshold of electrically-induced discharge had been lowered by the mucus, it could not be discerned. Intensity of discharge at high levels of electrical stimulation did not appear to differ from the controls. When pads of mucus from *Amphiprion percula*, *A. frenatus* and the anemone itself were used, results did not differ significantly from the controls.

It is quite clear that the presence of mucus from the partner fish did not raise the threshold of electrically-induced discharge of nematocysts. It is also interesting to note that maximum discharge in all cases was elicited within a narrow range of inductorium setting (4–5).

Discussion

The above investigations were principally directed toward understanding the physiological and behavioral mechanisms which maintain the animals in partnership and which protect the fish from the nematocysts of its host.

The reactions of unacclimated fish to the anemone were described in detail. These reactions differed considerably within our sample, but it must be remembered that the history of our nine fish was unknown to us. Some may have been free-living and some commensal with other species of anemone. However, the entire sample ultimately became acclimated to the Stoichactis. There is unquestionably a bond which attracts *A. percula* to this anemone and keeps the fish in it, once the acclimation process is completed. This process was first observed by Gohar (1948) who says (p. 39): "Fish of the commensal species may *develop* partnership with such anemones as *Discosomum giganteum* by cautiously approaching it. The

TABLE I

Threshold and intensity of discharge under faradic stimulation in isolated Stoichactis tentacles

Mucus pad	Control (mucus absent)				Fundulus			*A. percula*			*A. frenatus*	*Stoichactis*
Test	1	2	3	4	5	6	7	8	9	10	11	12
Induct.												
12	0	0	0	0	—	—	—	—	—	—	—	—
11	0	0	0	0	—	—	—	—	—	—	—	—
10	0	0	0	0	(+)	(+)	(+)	0	0	0	0	0
9	0	0	0	0	(+)	0	(+)	0	0	0	0	0
8	0	+	0	+	(+)	0	++	0	?	+	0	0
7	0	+	0	++	+	+	++	0	+	+	+	0
6	+	++	+	+++	+++	++	++	+	++	+++	+++	+
5	+++	+++	++	++++	++++	+++	++	++	+++	++++	+++	+++
4	++++	—	++++	—	++++	+++	++++	++++	++++	—	++++	++++

association is completed in one to a few days." Once the association is completed the bond is stonger; acclimated fish rarely wander away from the anemones, while unacclimated ones may wander all over the aquarium. In the course of the acclimation process we observed the "cautiousness" noted by Gohar. A fish touches the tentacles, often sticks at first and flees. But it *keeps coming back,* making more and more contact until no tentacular clinging occurs. We have not as yet identified those signs which attract the fish, beyond confirming Verwey's observation (1930) that the fish respond to visual cues. Since our anemone was kept in still water, part of the visual cue to an unacclimated fish may have been absent, since in nature the Stoichactis must be in almost constant motion in its shallow water habitat. We have not as yet investigated the possibility that specific chemical releasers from the anemone may be an important part of the bond. Tactile stimuli may also be important, for the fish appears to "seek" contact with the anemone during the acclimation process. The process of acclimation may be recognized by the action of the fish of bringing more and more of its body in contact with the host. The strength and effectiveness of stimuli from the anemone certainly affect the rate of attainment of the ultimate equilibrium between the partners, which is the consummation of the acclimation process.

The behavior of the anemone in relation to the fish was also carefully observed. Some writers have claimed that the commensals, even without contact, affect the behavior of the tentacles. Crespigny (1869) said (p. 10): " . . a Premnas now passes over the anemone and immediately the tentacula become erect and diverge, while their extremities become clubby. . . ." Herre (1936), working with the symbionts used in our investigation, says (p. 167): "But when an *Amphiprion* darted in among the beautiful but dangerous tentacles, they curled away from the intrepid invader." We have never observed any such action at a distance, in spite of efforts to elicit some response by bringing an acclimated Amphiprion, held by its lower jaw or immobilized in a plastic tube, within a fraction of a millimeter of the host. In the former test, water currents from the fish's pectoral fins gently waved the anemone's tentacles, but no such response appeared as that described above. Even when an acclimated Amphiprion was dragged across the disk, no response occurred which was not attributable to mechanical disturbance; we suspect that the observations of Crespigny and Herre were merely the result of water currents.

Gohar implies that the fish in some way affect the nerve net of the anemone when he speaks of the activity of the fishes appearing (p. 38) "as if they were . . . sympathetically caressing" closed anemones so that they opened. This observation was also made by Verwey (1930). Gudger feels that such behavior involves a certain "gentle massage." This activity may be effective in bringing the anemone back to its expanded state, and if so, then the fish is affecting the neuromotor apparatus of the host. But the purposive implication is unwarranted, since the behavior of the fish is probably not very different from that when the anemone is already expanded and may be under the control of the same stimuli as those eliciting typical "acclimating activity."

We have produced other evidence that the presence of the fish may affect the neuromotor apparatus of the host, for contact by an unacclimated fish may result in localized retraction of tentacles, and, if stimulation is particularly strong, a slight infolding of the disk may occur. This reaction is similar to that elicited by contact with prey, but is not as intense, involving briefer tentacular retraction and a weaker, more localized infolding of the disk. The frequently violent activity of the acclimated fish has no apparent effect on the anemone whatever. It is as though a physiological barrier had been set up during the acclimation process. We believe that the weak response of the anemone to an unacclimated fish indicates that the fish has not yet reached a state in which it fails to stimulate the host and that low intensity stimuli of the same nature as those received from prey are "getting through." It seems apparent that in the acclimation process, repeated contact with the anemone is necessary for the establishment of both the physiological protective barrier and the "bond of association" between the animals. We do not know the exact nature of the barrier. If one places a skinless piece of Amphiprion meat on the disk it is consumed, while a piece with skin attached is rejected and ultimately falls off the disk. Furthermore, if a piece of Amphiprion meat with skin and a piece of Fundulus meat with skin are placed side by side on the disk the former is rejected and the latter consumed. It appears as though the anemone "discriminated" between them. From this we are forced to conclude that a factor is present in the skin which affects the stimulus-response chain in the anemone. But the factor may do this indirectly by preventing nematocyst discharge, if, for instance, the normal feeding reactions depend upon the reception of information from receptors in the tentacles which are sensitive to bursts of nematocyst discharge, or to substances released from prey that has been "stung."

We are persuaded that the protection of the fish against its host's nematocysts does not involve a simple inability on the part of the fish to give adequate stimuli for discharge. We have shown that (1) the strongest stimulation of an isolated tentacle by a glass rod covered with Amphiprion mucus results in little or no discharge whereas like stimulation by a clean rod causes a burst of nematocysts; (2) when an Amphiprion is dragged across the disk of the host no discharge or adherence occurs; (3) the inner surface of a piece of Amphiprion skin sticks immediately to the tentacles, while the outer surface does not; (4) heat-treatment of the skin abolishes the protection; (5) a sponge covered with Amphiprion mucus will not stick to the tentacles, even when firmly pressed against them, while a clean sponge will; (6) heat-treatment of a mucus-covered sponge destroys the protection; (7) Amphiprion is immediately seized by another anemone (Anthopleura). All these observations argue for the existence of a heat-labile factor present on the outer surface of the skin of Amphiprion, which raises the threshold of discharge of nematocysts in the host Stoichactis.

What is the function of the behavioral process we call acclimation? We believe that this process, which other workers have suggested serves to change the condition of the anemone, is more probably a mechanism which changes the condition of the fish as the result of repeated contact between it and the anemone. It remains to be determined whether the fish has immunity to the nematocyst contents and whether acclimation has any relation to the maintenance of this immunity. There is a possibility, though we cannot offer conclusive proof, that acclimation may be related to changes in the mucus coat of the fish. Frequently, prior to complete acclimation, the protection of a fish is not perfect. Perhaps increasing contact with the anemone induces a greater general secretion of mucus or, specifically, more of the active principle in the mucus. Rough handling of the fish renders it susceptible to localized stinging, which may result from "breaks"in the protective mucus coat. During the early stages of acclimation the fin tips are the sites of nearly all localized clinging. These edges are precisely the areas which are first brought carefully into contact with the anemone. Similar clinging occurs at the site of a wound in a damaged fish, but after a short time, if the wound is not great, clinging no longer occurs. This may indicate the spread of mucus over the wound, renewing the integrity of the protective coat. It would seem that the characteristic fluttering movements of Amphiprion when on the disk of the anemone would be particularly effective in spreading mucus over the various sharp fin edges.

Clearly there remain many unsolved problems. It appears that the protective principle in the mucus coat takes its effect locally, is fast-acting and specific. It would be of interest to determine its rate of decay and to find out whether, after cessation of contact between a tentacle and Amphiprion mucus, there is some effect, however brief, on the threshold of nematocyst discharge. One would wish to know a good deal more about the chemical nature of the principle and whether it is present in other fishes such as Nomeus, the commensal of the Portuguese Man-of-War, Physalia.

SUMMARY

1. The behavioral process is described whereby the fish *Amphiprion percula*, after long isolation from the anemone Stoichactis, effects its association with the host.

2. This process appears to involve a gradual acclimation to the host, brought about by increasing contact with the host's tentacles. This appears to effect the establishment of both the "bond" and the physiological protective barrier between the animals.

3. Evidence is presented that an active principle is present in the mucus secreted on the outer surface of the integument of Amphiprion which raises the threshold of mechanically-induced discharge of the host's nematocysts. This factor does not affect the threshold of electrically-induced discharge. It is fast-acting, specific in its effect and heat-labile. It is not present in the muscle of the fish.

4. After contact between the host and an acclimated commensal no feeding reactions can be observed in the anemone such as occur when similar contact is made between Stoichactis and prey fish or between other anemones and Amphiprion. It is possible that this "inhibition" of the anemone may be the result of a direct effect on the nervous system by the active principle. However, it would seem more probable that this absence of feeding reactions even on violent contact may depend upon the fact that nematocysts are not discharged. Perhaps stimuli from receptors in the tentacles sensitive to nematocyst discharge or to substances from "stung" prey are necessary for the initiation of feeding reactions.

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A COMPARISON OF THE EFFECTS OF GOITROGENS ON THYROID ACTIVITY IN TRITURUS VIRIDESCENS AND DESMOGNATHUS FUSCUS[1]

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Salamanders of the genus *Triturus* have been used widely in studies of the histology, cytology, seasonal variation, and responses of the endocrine glands. Relatively few investigations of these matters have been made with members of other urodele genera. It would be interesting to know to what extent findings based on study of an exclusively aquatic newt are applicable to urodeles that are terrestrial in habit. There is some indication that the thyroid of *Triturus* may differ significantly in at least one respect from that of the terrestrial salamander, *Desmognathus,* namely, in the histological changes elicited by treatment with the goitrogenic drug, thiourea. Adams (1946) found that a high dosage and a long period of treatment are required to bring about hypertrophy and hyperplasia of the thyroid gland of *Triturus viridescens* whereas Fisher (1953) and Wheeler (1953) reported that the thyroid of *Desmognathus fuscus* responds much more readily and typically to this goitrogen. Since, however, the doses, environmental conditions, and length of treatment differed considerably in these studies, it is not possible to conclude with any assurance that *Triturus* is refractory to the effects of thiourea.

In our experiments a comparison is made of the responses of these two salamanders to treatment with two different goitrogens, thiourea and potassium perchlorate. The effects of these goitrogens on radioiodine uptake by the thyroid and on the histology of the gland were the basis for the comparison.

MATERIALS AND METHODS

Specimens of *Triturus* (*Diemyctylus*) *viridescens viridescens* (Rafinesque) were collected on October 20, 1955, from a pond near Monterey, Virginia. Specimens of *Desmognathus fuscus fuscus* (Rafinesque) were taken from beneath stones at the edge of a small stream on a thickly wooded hillside near Oliver Springs, Tennessee, over the period June 23–July 23, 1956. All animals were maintained in the laboratory for a minimum of two weeks before being subjected to experimental treatment. Both before and during treatment they were fed every other day, the *Triturus* with ground lean beef fortified with cod liver oil and calcium phosphate, and the *Desmognathus* with live meal-worm larvae. Throughout the experimental period the specimens were kept in a constant-temperature room at 19.0° ± 1.0° C.

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[1] Supported in part by AEC Contract No. AT-(40-1)-2000.

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[4] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

Goitrogens were injected into the body cavity on alternate days. Some animals were given injections of 0.1 ml. of a 1.0% aqueous solution of thiourea; others received injections of 0.1 ml. of a 0.2% aqueous solution of potassium perchlorate. Controls were given injections of 0.1 ml. of distilled water. The injections were made with a 27-gauge needle introduced into the body cavity through the muscles at the base of the hind leg. We chose this site to prevent the loss of fluid that sometimes occurs when injections are made directly through the abdominal wall. Successive injections were always given on alternate sides.

The uptake and turnover of radioiodine in the thyroids of experimental and control animals were observed by the following method: The animal to be studied was injected intraperitoneally with 0.1 ml. of 10% Holtfreter's solution containing 50.0 μc/ml. of I^{131}. At fixed intervals over a 2½-day period thereafter the animal was anesthetized in an aqueous solution of tricaine methane sulfonate (1 part in 1000) and the radioactivity of the thyroid and heart regions was measured by a scintillation counter consisting of a 1.5-inch NaI crystal cemented to the window of an RCA type-5819 photomultiplier tube and a conventional amplifier and binary scaler. The crystal and photomultiplier were mounted in a lead cylinder 5.2 cm. thick with a collimating slit measuring 4.0 by 12.0 mm. The ventrum of the anesthetized animal was apposed to the lead cylinder with the region to be counted directly over the slit.

The procedure just described was evolved from a series of preliminary experiments carried out to ascertain the optimal dosage of I^{131} for reliable counts, the time required for maximum uptake by the thyroid, the extent of individual variation in uptake among control animals, and the possible effects of frequent anesthetization on the uptake. In one preliminary experiment, counts were made at eight successive levels on the anterior-posterior axes of I^{131}-injected animals from the snout to the base of the tail, giving a profile of radioactivity. Relatively high counts were obtained in the region of the thyroid and in the abdomen. The heart region was selected as representative of the tissues in general (other than the gut) for comparison with the thyroid region. The counting on these two regions was done by centering the thyroid or the heart, as located with relation to external anatomical characters, over the collimating slit. During the experiment the mean background count was 1.68 counts per second. It did not vary significantly over the 60-hour counting period.

At the conclusion of the measurements of radioactivity the animals were killed. The lower jaws, containing the thyroid glands, were fixed in a solution containing equal parts of Bouin's solution and ethylene glycol monethyl ether. Sectioned thyroids were stained with Harris' hematoxylin and Ponceau de zylidine-orange II (Gray, 1952) for histological study.

The results to be described here were based on information obtained from the study of 72 salamanders divided into two series, one made up of animals that received injections for 30 days and one of animals that had 46 days of treatment. Each series consisted of six groups of six animals each; one group of control, one of thiourea-treated, and one of perchlorate-treated specimens for each of the two species.

Results

1. *Effects of thiourea and perchlorate treatment upon the histology of the thyroid*

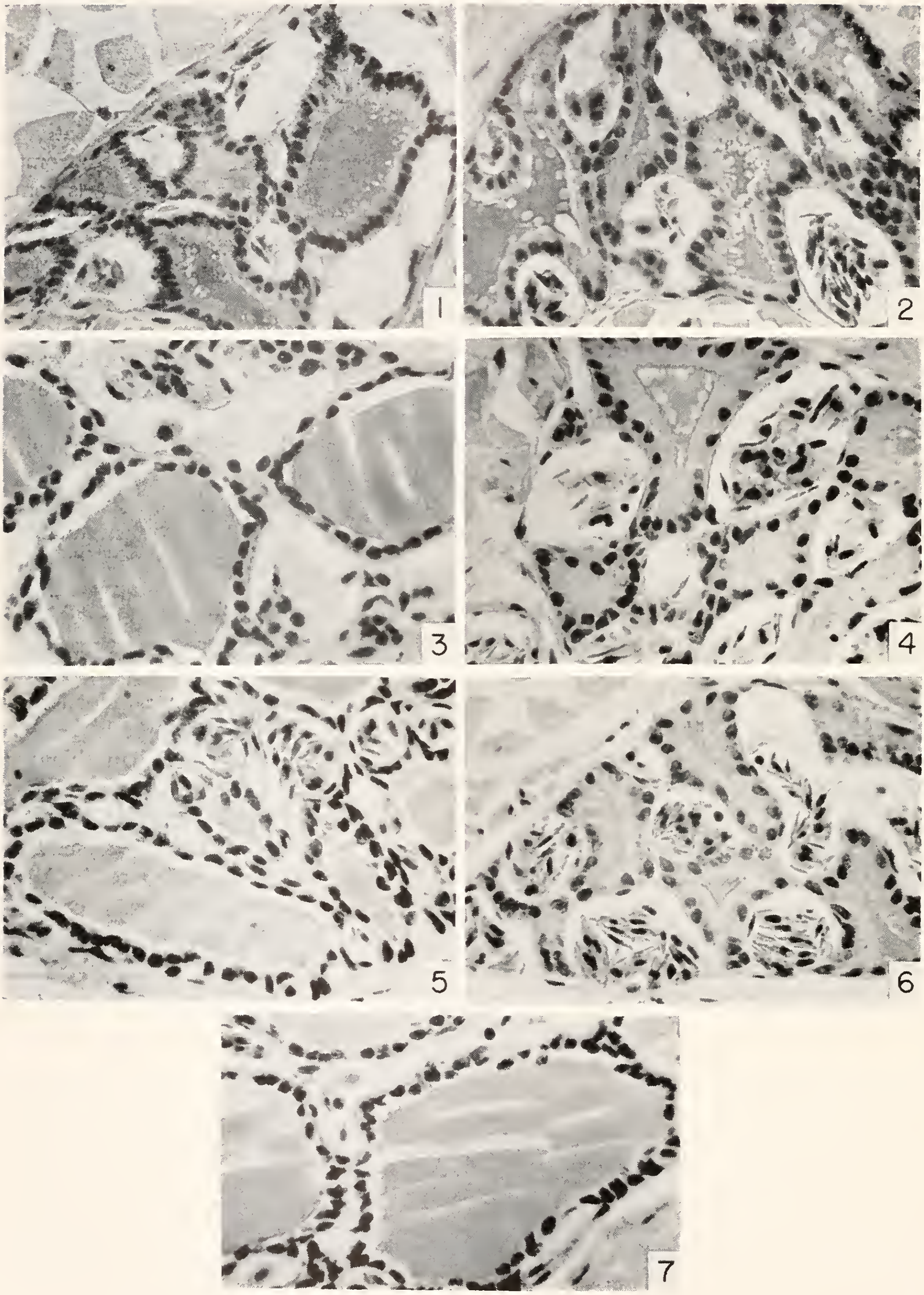
The thyroid glands of the control animals of the 30- and 46-day series did not differ significantly and will be described together. The thyroids of ten *Desmognathus* appeared to have moderate secretory activity. The follicles were relatively large, the colloid was homogeneous, and there was a moderate number of chromophobe droplets. The epithelium was cuboidal to low columnar. There was some but not a great deal of individual variation among the ten specimens. The extremes of variation in epithelial height, follicle size, and vacuolization of the colloid are shown in the photomicrographs (Figs. 1 and 2). The thyroids of ten *Triturus* controls presented a definite contrast with those just described. The follicles were larger, the epithelium much more flattened, and chromophobe droplets were either entirely absent or quite sparse. There was less individual variation in this group than in the *Desmognathus* controls and a single photomicrograph will suffice to illustrate the entire set (Fig. 3).

Examination of five pairs of thyroids of *Desmognathus* given treatment with thiourea for 30 days (15 injections) revealed a definite response indicated by a marked increase in height of the follicular epithelium, a folding of the follicle walls, a reduction in the amount of colloid present, and an increase in the number of chromophobe droplets. The thyroids of animals given this treatment for 46 days (23 injections) showed little difference from those just described except for a further reduction in the amount of colloid. A typical example is shown in Figure 4. The thyroids of *Triturus* given thiourea for 30 days or for 46 days were almost precisely like those of controls. The only definite change is an indication of hyperemia in the glands, the sections showing enlarged capillaries and many more blood corpuscles than were seen in controls (Fig. 5).

Potassium perchlorate treatment produced a still greater effect than thiourea treatment in *Desmognathus*. Even after 30 days, intrafollicular colloid was almost entirely lacking and the increase in epithelial height was quite striking (Fig. 6). Thyroids of animals treated with perchlorate for 46 days showed no significant further change, perhaps indicating that a maximal response had already been elicited. In *Triturus*, perchlorate was no more effective than thiourea in causing histological changes in the thyroid. In fact, there was not even an indication of hyperemia in the glands of the perchlorate-treated specimens; the glands were indistinguishable from those of controls (Fig. 7).

2. *Effect of goitrogens on uptake and release of I^{131}*

Table I gives the compiled results of measurements of radioactivity in thyroids of the animals of the 46-day series in comparison with radioactivity in their heart regions during a 60-hour period after injection with I^{131}. As has been pointed out, the measurements made in the heart region are presumed to be representative of the non-thyroidal tissues in general, except the gut, where iodine was being concentrated and eliminated. The measurements were corrected for the physical decay of I^{131} that followed the injection, and an analysis of variance was made of the means of the corrected counts.



Figures 1–7.

The least significant difference beween any pair of means in Table I was found to be 2.63 at the 5% level. The interactions among these individual means are apparently of considerable physiological significance and will be interpreted in the following section of this paper. It is well to note also that the differences between the over-all mean counts for the two species, for the two types of treatment and control maneuvers, for the two locations of counting, and for the six periods of counting were all highly significant in the statistical sense, *p* being less than 0.1% in each instance.

Discussion

1. *General comment and histological findings*

The histology of the thyroid gland of *Triturus viridescens* and the seasonal changes it undergoes have been described by Morgan and Fales (1942) who found that the thyroid is moderately active in early winter, gradually increases in activity during mid-winter and spring (up to the breeding season) but has low activity during the summer months. Our *Triturus* controls had thyroids that agreed closely with their description of the summer thyroid characterized by flattened epithelial cells and abundant homogeneous colloid. No comparable study of seasonal varation is available for *Desmognathus.* Our findings showed, however, that there was a definite difference in the histology of the thyroid in these two salamanders at the same season and under the same temperature conditions, *Desmognathus* showing histological indications of a much higher level of activity than *Triturus.*

The experimental use of thiourea as a goitrogenic agent is now well known. Although the precise details of its mode of action are still not completely understood, it is generally accepted that it does not interfere with the ability of the thyroid gland to concentrate iodide but does inhibit its ability to utilize iodide for hormone synthesis (Pitt-Rivers, 1950; Roche and Michel, 1955). As a result of this inhibition, the level of thyroid hormone in the blood of animals treated with thiourea falls, causing increased production of thyroid-stimulating hormone (TSH) by the pituitary. In turn, the elevation of the TSH level induces hypertrophy, hyperplasia, and hyperemia in the thyroid and the release of its intrafollicular colloid. The "goitrogenic" effects of thiourea thus result from the pituitary stimulation rather than from the direct action of the drug itself. In our experiments, the 30- or 46-day treatments of *Desmognathus* with thiourea brought about all these structural changes in the thyroid. *Triturus*, similarly treated, showed no response

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Figures 1 and 2. Sections of thyroid glands from control specimens of *Desmognathus* killed in July. Histological evidence of moderate secretory activity ranges from the condition shown in Figure 1 to that shown in Figure 2.

Figure 3. Thyroids of *Triturus* killed in July gave a uniform appearance of inactivity as illustrated in this section.

Figure 4. Section of thyroid gland from *Desmognathus* treated with thiourea. Marked secretory activity is indicated.

Figure 5. Thyroid section from *Triturus* treated with thiourea.

Figures 6 and 7. Thyroid sections from *Desmognathus* (6) and *Triturus* (7) treated with potassium perchlorate. Note marked response shown in *Desmognathus* and lack of response in *Triturus.*

aside from an increase in the vascularity of the gland. This lack of response in *Triturus* agrees with the findings of Adams (1946) who immersed specimens of *Triturus viridescens* in thiourea solutions that were increased in strength over a period of 42 days to 0.528% and then allowed the animals to remain in this concentration for 44 days longer. Such animals showed no change in thyroid histology as compared with controls. Adams found that animals treated similarly with solutions of twice this strength did show an increase in epithelial height and a reduction in colloid, indicating that a sufficiently high dosage may produce a response. Since, however, only two of the ten animals so treated survived to the end of the experiment, it seems clear that this concentration is quite toxic and the changes in thyroid structure may be related to this toxicity. Fisher (1953) reported histological changes in the thyroid of *Desmognathus* after thiourea administration that are in complete accord with our findings. His animals were treated by immersion in a 0.05% solution of thiourea and, although this is only one-tenth as strong as the weakest solution used by Adams, it caused hyperplasia, loss of colloid, and hyperemia—all of which became apparent after only two weeks and had increased markedly by six weeks. Although Fisher's experiments were performed in January and February and the season at which Adams' work was done is not given, the results of these two studies seemed to indicate, as did our findings, that the structure of the thyroid of *Triturus* was relatively unaffected by treatment with thiourea whereas that of *Desmognathus* was markedly altered.

The effects of perchlorate have apparently not been investigated previously for either of these animals. Studies on mammals indicate that the action of perchlorate is quite unlike that of thiourea, for it interferes with the process of iodide concentration (Wyngaarden, Wright and Ways, 1952). The thyroids of animals given effective doses of this drug are unable to accumulate iodine from the plasma. This, of course, prevents synthesis of thyroid hormone (TH) and, just as in thiourea treatment, the resultant lowering of the TH level and rise in TSH level cause the histological changes in the thyroid that are usually associated with a high rate of secretory activity. In our experiments the histological results of perchlorate treatment precisely paralleled those obtained with thiourea. The *Desmognathus* thyroid was strikingly affected but that of *Triturus* remained unchanged.

Desmognathus, then, after treatment with either of these two goitrogenic drugs, gives indication of the increased TSH production, which would be expected on the basis of the work with mammals. *Triturus* shows little or no sign of any increased TSH level. The lack of response in *Triturus* could be explained in at least two different ways. One possibility is that this salamander is refractory to the drugs so that, at the dosage level used, thiourea and perchlorate both failed to inhibit hormone synthesis. If this were the case, the treatment would have caused no lowering of the TH level in the blood; therefore, no stimulation of the pituitary to increased TSH production would have occurred. The other possibility is that the drugs were effective in preventing hormone synthesis but that the pituitary failed to respond in the usual way to the TH decrease. Then no increase in TSH would occur and none of the effects of this hormone on the thyroid would be observed. The results of the study of radioiodine uptake will serve to indicate which of these alternatives is correct.

2. *Iodine uptake in control animals*

It was observed (Table I) that in the *Desmognathus* controls the counts in the thyroid region were not significantly higher than those in the heart region at 6 hours after injection of I^{131}. By 12 hours, however, they had risen to a high level. They then declined steadily, reaching about one-half the peak level by 2.5 days, but remained significantly higher than the counts in the heart region throughout this period. *Triturus* controls, on the other hand, showed neither an initial difference in counts between thyroid and heart nor a steady decline in the counts of the thyroid region during the 2.5-day period, although the counts at the heart level declined steadily as I^{131} was eliminated from the body, with the result that the thyroid counts were significantly higher than the heart region counts from 24 hours on. Comparison of the thyroid counts with heart counts showed that radioiodine was taken up actively, reached a high level, and was rapidly released by the thyroid of *Desmognathus* whereas in *Triturus* the gland accumulated I^{131} more sluggishly and in lesser amounts and the turnover (during 2.5 days) was negligible. These results are in accord with the histological findings, which indicate a greater physiological activity of the thyroid in untreated *Desmognathus* than in untreated *Triturus.*

3. *Iodine uptake in thiourea-treated animals*

Table I shows that *Desmognathus* treated with thiourea had a high initial uptake of I^{131} by the thyroid (falling in the same range as the peak for the controls) and then a rapid loss; the differences between thyroid and heart regions were there-

TABLE I

Radioactivity (corrected for physical decay) in counts per second of specimens of Triturus viridescens and Desmognathus fuscus at the indicated periods of time after injection with 5 μc. of I^{131} after 46 days of treatment with goitrogens. Each number represents the mean of counts made on six specimens. The least significant difference (at the 5% level) calculated for these means is 2.63

Species	Time after I^{131} inj. hours	Control animals		Animals treated with thiourea		Animals treated with $KClO_4$	
		Region of thyroid	Region of heart	Region of thyroid	Region of heart	Region of thyroid	Region of heart
Triturus viridescens	6	10.30	10.94	9.64	9.96	10.25	11.68
	12	11.84	9.42	9.90	9.41	8.77	9.85
	24	11.70	8.59	8.90	8.80	5.96	6.22
	36	11.57	7.76	8.86	8.56	5.04	5.03
	48	11.14	7.47	8.79	8.06	3.92	4.06
	60	11.11	7.89	9.10	8.11	3.53	3.95
Desmognathus fuscus	6	8.68	6.80	10.22	6.84	6.40	6.95
	12	12.13	5.66	8.42	6.23	5.58	6.05
	24	10.98	4.56	6.39	4.13	4.67	4.43
	36	10.94	3.78	4.51	3.58	3.91	3.80
	48	8.73	3.64	4.15	3.16	3.14	3.33
	60	6.86	3.35	3.71	3.09	2.84	3.03

fore quite significant at 6 hours after injection of I^{131} but not significant thereafter. In *Triturus,* similarly treated, the iodine content of the thyroid was different from that of the controls only in the 24- and 36-hour counts and the thyroid region showed no count significantly higher than that of the heart region at any time.

These results may be interpreted as follows. It is clear from the histological study that the thiourea treatment caused a hypertrophy of the thyroid in *Desmognathus* and one may assume that this is accompanied by a high affinity for iodine. This would account for the initial high uptake of I^{131}. Since, however, the gland was unable to bind iodine, because of the treatment with thiourea, and also since the TSH level in the blood was presumably high, the I^{131} that had been taken up was very rapidly lost. In *Triturus* the thyroid did not hypertrophy and therefore would not be expected to have a high affinity for iodine. Indeed, it takes up I^{131} and retains it to about the same degree as the controls. Not being able to bind I^{131} the thyroid releases it, but since the TSH level is apparently unaffected by treatment with thiourea in *Triturus,* the release of I^{131} from the thyroid is much slower than in *Desmognathus.*

4. *Iodine uptake in animals treated with potassium perchlorate*

Treatment with perchlorate produced the same effect on radioiodine uptake in both salamanders. Counts in the thyroid region did not differ from those in the heart region at any time. Indeed, the counts over the thyroid were, in general, slightly lower than those over the heart, probably because of the difference in the volume of circulating blood in the two regions. In any case it seems clear that perchlorate was equally effective in these two animals in preventing any accumulation of I^{131} by the thyroid.

In perchlorate-treated *Triturus,* the counts in the heart region declined more rapidly and reached a significantly lower level than in the control or thiourea-treated *Triturus.* This indicates that elimination of I^{131} from the body goes on rapidly when iodide uptake by the thyroid is blocked but is in some way delayed when the thyroid is actively concentrating iodide. This delay occurs regardless of whether the iodine taken up by the thyroid is ultimately bound to protein. It is well known that, so long as there is no interference with the thyroid's ability to concentrate iodide, the gland acts as an iodine reservoir, continually taking up iodine from the plasma and passing it back at a slow but steady rate so that the decline in iodine content of the plasma caused by elimination from the body is partially compensated by the exchange of iodine with the thyroid. In perchlorate-treated animals, where no such mechanism is in operation, the rate of decline in the iodine content of the plasma would depend solely upon the rate of iodine excretion. It is noteworthy that the rate of decline in the heart-region counts in *Desmognathus* is essentially the same in all three groups. The failure of the thyroid to show any iodine reservoir effect in this animal, even when its iodide-concentrating ability is unimpaired, is probably to be ascribed to the high activity of the gland. In thiourea-treated *Desmognathus,* although the initial uptake of radioiodine was high, I^{131} was very rapidly passed out to the plasma and, since none was bound, the decline in uptake in both thyroid and plasma was rapid, paralleling that of perchlorate-treated *Desmognathus,* where no iodide-concentration occurred at all. It may be assumed that in control *Desmognathus* any unbound iodine is also rapidly exchanged with the plasma, and the counts in the heart region again fall rapidly. Undoubtedly, the counts in the thyroid region remain significantly high in the controls because in these animals, in contrast with the thiourea-treated specimens, a part of the iodine is bound to protein and is thus unavailable for ready passage into the plasma.

5. *Possible adaptive significance of findings*

The results of the study of radioiodine uptake indicate that the differences in histological response of the thyroid in *Triturus* and in *Desmognathus* should not be attributed to a difference in the direct effects of the goitrogens on thyroid functioning in the two. Perchlorate treatment definitely prevented thyroidal build-up of I^{131} in both, and there is good reason to conclude that thiourea treatment interfered with binding of I^{131} in both. It would seem, therefore, that the lack of histological change in the *Triturus* thyroid, as well as its normally inactive state, must be attributed to some unusual condition in the pituitary, involving a low level of TSH production. Such an explanation has been suggested to account for what is apparently a similar situation in the goldfish (*Carassius auratus*). The goldfish thyroid exhibits a histological appearance of very low activity throughout the year and at no season (Fortune, 1955) undergoes hypertrophy after administration of thiourea (or other goitrogens). It does, however, respond very strongly to injected TSH both histologically (Gorbman, 1940) and physiologically (Berg and Gorbman, 1954). Fortune (1956) suggests that the inactive thyroid of the goldfish (possibly acquired through many generations of artificial selection) may be an important adaptation permitting its tolerance of an exceptionally wide range of temperatures (0° to 41° C.) and cites the rise in thermal death point from 23° C. to 33° C. in the teleost *Phoxinus laevis* after treatment with thiourea as supporting evidence for his suggestion.

This hypothesis may also apply to *Triturus viridescens*. Although no specific data are available concerning the temperature range of this species, it is undoubtedly quite wide. The animal lives in pools and ponds in both wooded and open situations and has a geographical range from southeastern Canada to Georgia and Alabama. It is certainly able to survive successfully in ice-covered water, and the water temperature in unshaded ponds in mid-summer must frequently rise above 30° C. *Desmognathus,* on the other hand, lives in moist situations usually in close proximity to small streams in deep woods. In our experience it does not survive in the laboratory at temperatures above 25° C. and does best below 20° C. Several rather obvious devices for testing this theory come to mind. One, namely, observation of the effect of TSH administration on iodine metabolism in *Triturus,* is currently being employed by us.

The authors wish to acknowledge the assistance of Dr. A. W. Kimball of the Oak Ridge National Laboratory Mathematics Panel in the statistical analysis.

SUMMARY

1. Specimens of *Triturus viridescens* and *Desmognathus fuscus* were injected on alternate days with 0.1 ml. of 1.0% thiourea. Others were injected on alternate

days with 0.1 ml. of 0.2% potassium perchlorate. Histological study was made of the thyroid glands of both experimental and control animals after 30 days and 46 days of treatment. Measurements of uptake and turnover of injected I^{131} were made on the animals treated for 46 days.

2. Evidence was obtained from the histological observations and from the use of radioiodine to show that although the thyroids of control specimens of *Desmognathus* were physiologically active, those of *Triturus* controls were rather inactive.

3. Both thiourea and potassium perchlorate inhibited thyroidal function in *Desmognathus,* as evidenced by both histological changes and changes in radioiodine uptake.

4. In *Triturus,* thiourea brought about only a slight hyperemia and potassium perchlorate produced no detected histological change in the thyroid. Radiological measurements after the injection of I^{131}, however, indicated that the same physiological responses taking place in *Desmognathus* also occurred in *Triturus* but at a lower level of thyroidal function.

5. Measurements of radioactivity in the heart region demonstrated that iodine was readily excreted from all the specimens of *Desmognathus* and from the individuals of *Triturus* treated with potassium perchlorate. Elimination of iodine was relatively slow in the other specimens of *Triturus.*

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RESPONSE OF THE MALE REPRODUCTIVE SYSTEM OF LIZARDS (ANOLIS CAROLINENSIS) TO UNNATURAL DAY–LENGTHS IN DIFFERENT SEASONS

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In this study we have examined the response of the male reproductive system of the green anole, *Anolis carolinensis,* to unnatural day-lengths at different seasons of the year. Evidence of the photoperiodic control of reproduction in reptiles has been slowly accumulating. Burger (1937) found that artificially increased day-length stimulated a new spermatogenic cycle in the red-eared turtle, *Pseudemys scripta elegans.* Clausen and Poris (1937) reported unseasonal gonadal hypertrophy and spermatogenesis in green anoles exposed daily to 18 hours of light. Bartholomew (1950, 1953) described similar gonadal recrudescence in the desert night lizard, *Xantusia vigilis,* maintained at 16-hour day-lengths.

The above investigators have been primarily interested in the condition of the gonads. Descriptions of accessory sex organs have not been detailed enough to allow for a positive statement that reptiles can be brought into full breeding condition by artificially increasing day-length. In the experiments to be described we have examined both gonads and certain accessory sex organs to obtain a more complete measure of breeding condition. Further, the influence which the condition of the reproductive system at the beginning of an experiment has upon its response to day-length alterations has not been thoroughly examined in all seasons. We have studied the effect of short days prior to and during the breeding season, as well as the effect of long days during and following the breeding season. To our knowledge there has been no previous effort to determine whether a seasonal "refractory period," characteristic of passerine birds (for review see Hammond, 1954), is present in reptiles or if a period of exposure to short days is necessary before a new reproductive cycle can be initiated (Miller, 1954).

METHODS

Animals were collected as needed over a period of three years in the vicinity of New Orleans. Adult animals (61 to 71 mm. snout-vent length), that had probably been through a previous reproductive period, were separated from immatures or sub-adults (51 to 60 mm.). The latter had not been through a previous complete reproductive hypertrophy but were due to become sexually mature in the next normal breeding season (Fox, 1958).

Methods of animal care have been reported previously (Fox and Dessauer, 1957). Cage temperatures were maintained at 28 ± 2° C. Artificial lighting was supplied by means of daylight fluorescent lamps mounted above the cages. These were regulated by automatic time switches to supply 18L (hours of light per

24-hour period), 16L, 14L, 9L or 6L. Animals exposed to natural day-length were placed in front of a north-facing window.

Experiments were conducted for approximately 60 days unless otherwise stated. At the end of each experiment lizards were killed with ether, and their fat bodies, liver and left testis weighed. Loss of body weight during the course of an experiment, and liver and fat body weights at autopsy were useful in estimating the degree of starvation of unresponsive lizards. The right testis with attached epididymis, the ductus deferens, and the right kidney were fixed in Bouin's solution, embedded in paraffin, sectioned at 10 micra, and stained with Harris' hematoxylin and eosin. The state of spermatogenesis and the cytology of interstitial cells and accessory sex organs were studied microscopically. Measurements of the diameter of seminiferous tubules, the diameters and epithelial heights of the ductus epididymidis, ductus deferens, and the sexual segment of the kidney were made with a calibrated ocular micrometer. All mensural data were plotted as histograms and subjected to the "*t* test" for possible significance. The size of each sample, mortality and number of starving animals (fat bodies less than 1% of body weight) are presented in Table II. Column "N" in Table II is the number used in calculating standard deviations and "*t*" values for determining the level of significance of the data when it seemed justifiable to eliminate the measurements on starving animals. Differences are accepted as significant at the 5% level and highly significant at the 1% level of probability.

In reporting the results, frequent reference is made to arbitrarily delimited stages of the normal reproductive cycle of wild male *Anolis*. These stages are characterized briefly in Table I; detailed descriptions are presented by Fox (1958).

Although considerable effort was made to locate and measure interstitial cells of the testes, it was felt that these data were unsatisfactory for valid statistical analyses. Data on the sexual segment of the kidney, however, afford an index to androgenic activity (Reynolds, 1943). The sexual segment of *Anolis*, like that of *Sceloporus* (Forbes, 1941), includes the entire set of uriniferous collecting ducts and ureter (Fox, 1958). Measurements were taken in the distal, middle, and proximal regions of the collecting ducts, but only those of the distal end are recorded in Table II.

RESULTS

Experiment 1. September–November: Adult lizards exposed to 18L and natural day-length

At the beginning of the experiment adults had just completed a breeding season and the gonads and accessory sex organs were in their most atrophic state

TABLE I

Arbitrary stages in the normal reproductive cycle of Anolis

	Spermatogenesis	Accessory sex organs
Stage I	Dividing spermatogonia	Atrophic
Stage II	Primary spermatocytes predominate	Slight hypertrophy
Stage III	Maximum development; large numbers of spermatocytes and spermatids	Near maximum hypertrophy
Stage IV	Large numbers of spermatids; spermatocytes reduced	Maximum hypertrophy
Stage V	Only spermatids numerous	Partial atrophy
Stage VI	Expulsion of all active cells	Atrophic

TABLE II

Experiment	Light exposure	Number	Mortality	Starving	N	Seminiferous tubule diameter μ	Spermatogenic stage						Ductus epididymidis epithelial height μ	Ductus deferens diameter μ	Sexual segment epithelial height μ
							I	II	III	IV	V	VI			
1 Sept.–Nov. Adults	18L	19	2	2	15	120–290† 206±48.9††	2	9	4				11–35† 20.0±6.7††	40–100† 59±15.1††	13–22† 16.1±2.9††
	ND*	14	0	0	14	50–170 110±26.2	14						8–13 10.8±1.5	40–60 53±8.0	13–16 14.0±1.1
2 Sept.–Nov. Immatures	18L	33	12	2	21	90–280 174±57.6	7	9	5				10–30 18.8±6.1	40–140 62±27.2	13–25 17.7±3.6
	ND	16	5	2	11	70–170 107±31.6	11						10–16 13.7±2.1	30–60 48±7.7	13–22 16.9±2.7
3 Oct.–Dec. Immatures	18L	45	9	6	30	90–290 173±62.0		9	15	6			8–32 18.4±8.4	50–180 81 ±38.0	16–38 23.3±5.0
	16L	14	5	3	6	90–260 156±67.5		2	1	3			12–38 21.7±10.5	50–210 120±68.8	16–32 20.4±3.5
	9L	29	9	3	17	80–190 127±29.2	10	7					6–13 11.6±2.1	40–70 54±8.8	13–25 19.1±2.3

* Natural day-length (maximum = 14 hours, minimum = 10 hours).
† Range.
†† Mean and standard deviation.

TABLE 11—*Continued*

Experiment	Light exposure	Number	Mortality	Starving	N	Seminiferous tubule diameter μ	Spermatogenic stage						Ductus epididymidis epithelial height μ	Ductus deferens diameter μ	Sexual segment epithelial height μ
							I	II	III	IV	V	VI			
4 Nov.–Jan. Adults	18L	26	5	3	18	110–300 245±50.1	1	1	10	6			12–30 25.6±6.6	90–220 167±45.3	20–50 35.8±9.8
	ND	21	0	8	13	90–280 176±56.1		9	4				10–18 13.1±2.4	30–60 45±12.6	12–20 16.9±2.6
5 Dec.–Feb. Adults	18L	8	4	2	4	240–305 271±31.2			1	3			22–29 24.8±3.4	67–130 106±30.6	17–32 22.5±6.6
	16L	26	6	2	18	240–310 269±15.5			5	13			19–38 27.6±6.6	100–190 150±30.8	25–50 37.6±8.1
	9L	20	0	4	16	230–300 269±24.3		1	11	4			16–35 22.5±4.8	80–230 142±52.4	16–40 24.9±7.6
6 Jan.–March Immatures	18L	26	6	2	18	160–330 256±38.1			3	13	2		21–38 30.0±6.6	120–300 185±49.5	25–51 37.3±8.7
	9L	25	15	3	7	180–300 246±40.9		1	5	1			18–32 22.4±4.9	100–210 142±38.9	21–32 26.0±3.9

TABLE II—*Continued*

Experiment	Light exposure	Number	Mortality	Starving	N	Seminiferous tubule diameter μ	Spermatogenic stage						Ductus epididymidis epithelial height μ	Ductus deferens diameter μ	Sexual segment epithelial height μ
							I	II	III	IV	V	VI			
8 May–July Adults	18L	10	4	0	6	176–300 212±48.3			1	3	2		16–32 24.0±5.4	70–192 149±49.4	13–32 22.0±8.2
	ND	11	2	1	8	210–262 239±17.4			1	7			19–36 28.3±4.9	160–196 179±12.6	22–51 37.0±9.1
	9L	8	4	0	4	77–120 100±18.7						4	9–16 13.4±2.7	50–85 63±15.4	12–17 13.7±1.8
9 July–Sept. Adults	18L	22	3	2	17	112–290 222±40.2	2	3	7	5			13–32 23.9±6.6	77–215 150±42.5	18–51 33.3±10.1
	ND	10	0	0	10	55–118 81±22.1						10	10–20 14.9±3.6	40–96 70±20.1	13–20 15.9±2.5
10 Aug.–Oct. Adults	18L	15	1	3	11	80–250 189±70.1	2	1	4	4			10–35 24.5±8.7	50–230 178±70.7	13–35 22.3±7.9
	14L	28	7	3	18	70–280 146±71.1	5	10	3				10–38 21.2±9.2	60–150 100±21.4	13–26 18.1±4.1
	ND	33	6	0	27	75–135 120±17.9	27						12–23 16.1±2.9	45–75 62±9.5	12–19 15.9±2.5

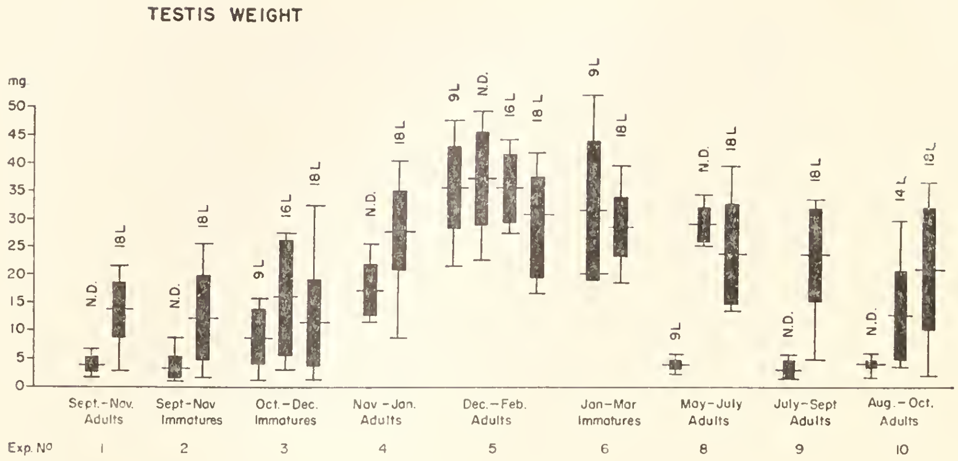

FIGURE 1. Testis weights demonstrating the seasonal gradient in testicular response to exposure to long photoperiods. The terminal horizontal bars indicate the range in sample weights, the middle horizontal bar indicates the sample mean and the solid rectangle represents the standard deviation plotted on both sides of the mean. N. D. = exposure to natural day-lengths; L = hours of artificial light per 24 hours.

(end of Stage VI). By the end of the experiment, animals in the wild had initiated spermatogonial mitoses but their accessory sex organs were still atrophic (Stage I). The 18L sample underwent a highly significant increase in testis weight (Fig. 1) although two apparently healthy animals did not respond to the light treatment. Control animals retained small gonads that averaged significantly less in weight than those of wild animals in November (Fig. 2). Seminiferous tubule diameters were greater and spermatogenesis more advanced in the experimental sample (Table II). Whereas none of the control animals advanced further than spermatogenic Stage I, four of the experimentals were classified as early Stage III. The total cell number, however, was about half that present in a normal Stage III gonad.

The accessory sex organs were partially stimulated by exposure to 18-hour day-lengths at this season (Table II). The ductus epididymidis underwent a very significant increase in diameter and epithelial height. In half of the sample the cells contained secretion granules (Stages II and III); in six animals sperm were present in the lumen (beginning of Stage III). Secretion granules and sperm were lacking in all the controls (Stage I). Neither the average of epithelial height nor diameter of the ductus deferens significantly increased in the 18L sample (Table II). In three animals, however, sperm had entered this organ and it was significantly enlarged, convoluted and contained secretion granules (Stage III). The ductus deferens of the controls remained atrophic (*viz.*, empty, not convoluted, and with a pseudostratified agranular epithelium).

Measurements of the sexual segment (Table II) did not reveal significant differences between the means of the experimental and control samples. Four experimental animals, however, showed slight hypertrophy and the presence of eosinophilic granules at the tips of the epithelial cells of the ureter and the distal ends of the collecting tubules (Stage II). No hypertrophy or secretion occurred among the controls.

Experiment 2. September–November: Immature lizards exposed to 18L and natural day-length

Although the reproductive organs of these young animals had achieved a partial maturity during the previous summer, they were completely atrophic at the beginning of the experiment. Wild animals in this age group were at minimal reproductive development at the close of the experiment (Stage I).

Immature lizards on 18L underwent a highly significant gonadal response. Testis weights were markedly greater ($P < 1\%$) than the controls (Fig. 1). Four were more responsive to the light treatment than adults during the same period. Spermatogenesis (Table II) almost reached peak activity (Stage III) in a few individuals, whereas none of the controls progressed beyond Stage I.

Of the accessory sex organs studied, only the ductus epididymidis proved to be significantly hypertrophied ($P < 1\%$) by the long light treatment. Over half the animals showed secretory granules and one-third had sperm in the epididymis. In all controls the epididymis was atrophic and without sperm. Three experimental animals showed enlargement of the ductus deferens (Stage II). They had a few sperm in the lumen, the epithelium was taller and the cytoplasm granular. The ductus deferentia of the controls were atrophic (Stage I).

Five experimental animals showed a slight hypertrophy of the sexual segment of the kidney with secretion granules at the tips of epithelial cells near the ureter (Stage II). The sexual segment of a few controls underwent a minor hypertrophy but no signs of secretion were present.

Experiment 3. October–December: Immature lizards exposed to 18L, 16L and 9L

At the beginning of this experiment the animals were in a stage of minimal development. The healthy animals on 16L and 18L showed a highly significant increase in testis weight over those kept at 9L (Fig. 1). Animals that lost weight did not respond to the light treatment. Many kept on the long days had very large seminiferous tubules and had progressed into Stages III and IV of spermatogenic development (Table II), without achieving, however, the high volume of sperm production characteristic of adults during the breeding season. Nearly half of the 9L animals attained the spermatogenic development of adult wild animals for December (Stage II), the others remained in Stage I.

Both samples on 18L and 16L showed highly significant or significant hypertrophy of the various accessory sex organs when compared to those on 9L. The differences between the 18L and 16L samples were not statistically significant. The epididymis of the animals on long days contained many sperm. The cytoplasm of the ductus epididymis was granular and the cell height and diameter much enlarged (Stages II and III). The epididymes of controls were all in Stage I. Significant numbers of animals on long day treatment had sperm in the ductus deferens. In these individuals the diameter of the ductus was increased, the epithelium hypertrophied and the cytoplasm granular (Stage III). A few animals remained in Stage I or II.

The sexual segment underwent a significant hypertrophy on the 18L ($P = 1\%$) and 16L ($P = 5\%$) regimes. Secretion occurred in only five animals on 18L and one on 16L. In these animals the epithelium of the ureter and the distal end of the collecting tubules exhibited eosinophilic granules in the apical $\frac{1}{2}$–$\frac{2}{3}$ of the cells (Stage II). The 9L series showed only slight hypertrophy, and no signs of secretion (Stage I).

Experiment 4. November–January: Adult lizards exposed to 18L and natural day-length

During November the reproductive system of *Anolis* is in Stage I. Lizards on 18 hours of day-length underwent marked hypertrophy of the testis ($P < 1\%$) compared to controls on natural day-length (Fig. 1). The testes of the controls enlarged also, but the sample mean did not differ significantly from that of wild animals killed in January (Fig. 2).

Most of the experimental animals reached the peak of seminiferous tubule diameter and spermatogenic development (Stage III). A few passed the peak and advanced to Stage IV. Four control animals progressed to early Stage III but these were far from attaining peak development. The remainder of the controls were in spermatogenic Stage II.

The accessory sex organs of the experimentals were greatly hypertrophied ($P < .01$; Table II). In most of the sample the ductus epididymidis was filled with sperm and the epithelial cytoplasm completely filled with eosinophilic granules (Stages III and IV). Four controls had a secretory epididymis with a few sperm in the ductus (Stage II). The ductus deferentia of the 18L sample were very convoluted, filled with sperm and highly secretory (Stages III and IV). Those of the control sample were largely atrophic and empty (Stages I and II).

The sexual segment of the experimentals was hypertrophied (Table II) and highly secretory at its distal end in all animals (Stage II) and in its middle portion in half the sample (Stage III). Control animals showed no signs of secretion although wild animals at this time show slight activity (Stage II).

Experiment 5. December–February: Adult lizards exposed to 18L, 16L and 9L

During this period the reproductive system of wild animals progresses through Stage II with some individuals reaching early Stage III. The distribution and sample means of testis weights were very similar for animals exposed to 9L and 16L (Fig. 1). Testes averaged smaller in the 18L sample. There were no significant differences in seminiferous tubule diameters (Table II). All healthy animals in the 16L and 18L series reached peak spermatogenic development (Stage III), and two-thirds passed on to Stage IV. Animals in the 9L sample were largely in Stage III, but a few merited assignment to Stage IV. When subjected to the Chi-square test, the 16L and 18L samples proved to be very significantly advanced beyond the 9L sample.

In the 16L sample, but not in the 18L sample, the epithelial cells of the ductus epididymidis were significantly taller than those of the 9L sample. Diameters of both the ductus epididymidis and ductus deferens were similar in the 9L and 16L samples, but averaged significantly smaller in the 18L sample. All were secretory and contained sperm. The ductus deferens appeared to be more highly convoluted in the 16L sample than in the 9L sample, indicating greater sperm storage. Those of the 18L animals were less convoluted and contained considerably fewer sperm than either of the two other samples.

The sexual segment was hypertrophied in both the 9L and 16L samples. In the 16L sample Stage III was reached whereas in the 9L sample the sexual segment remained in Stage II. The distal ends of the collecting tubules were hypertrophied and secretory in both samples. The middle portions of the collecting tubules were secretory in thirteen animals on 16L but in only one on 9L. All sexual segments of the 18L sample were atrophic except that of one animal in which the distal end was secretory.

Data on testes weights were available for a series of captive animals exposed to natural day-length during this period. The mean testis weight was comparable to that found in the 9L and 16L series (Fig. 1).

Experiment 6. January–March: Immature lizards exposed to 18L and 9L

At the beginning of this experiment smaller immature male lizards were in Stage I and larger ones in Stage II. At the end of this experiment the mean testis weight of the 9L sample was greater (Fig. 1), although not significantly so, than the 18L sample. However, animals on 18L were far advanced beyond those on 9L in respect to spermatogenesis (Table II). Most of them appeared to have passed the peak of gonad development (Stage IV).

The ductus epididymidis was secretory and filled with sperm in both samples. Epithelial height of the 18L sample (Table II) was significantly greater. The ductus deferens was secretory, convoluted and filled with sperm in most animals of both samples. Those of the 18L sample had a significantly greater diameter, appeared to be more convoluted and to contain more sperm. In three animals of the 9L sample the ductus deferens was pseudostratified, non-secretory and contained very few or no sperm.

The sexual segment was very significantly hypertrophied in the 18L sample. In over half of this sample it was secretory in the ureter and distal ends of the collecting tubules, and in ⅓ it was secretory through the middle portion (Stage III). Most sexual segments in the 9L sample remained in Stage I; in two animals they were secretory through the middle segment (Stage III) and in one secretion occurred in the ureter and distal end only (Stage II).

Regardless of the length of light exposure, most animals that underwent marked testicular development and hypertrophy of the accessory sex organs were over 55 mm. snout-vent length at the start of the experiment and grew to over 60 mm. during the two months period.

Miscellaneous winter experiments

Data available on a few animals maintained for longer than the standard 60-day period are pertinent to this study. Eight immature lizards kept at 9L from December 10 to April 4 underwent considerable hypertrophy of the reproductive system despite the short day-length. Testes ranged from 8 to 33 mg. with a mean of 22 mg. Spermatogenesis reached Stages III and IV in the larger gonads.

These same individuals had sperm in the ductus epididymidis and ductus deferens, both of which were hypertrophied and secretory. The sexual segment of two larger and faster growing individuals was secretory through the middle portion (Stage III). Those lizards that reached 60 mm. snout-vent length had the largest gonads and the most hypertrophied accessory sex organs. In those less than 60 mm. the extent of testicular hypertrophy was closely correlated with body growth.

Four animals of the same size group survived 18L exposure during the above period. The range in testicular size (3 to 33 mg.) was similar to that in the group exposed to 9L but the gonads averaged considerably smaller (16.5 mg.). Only two possessed large gonads with spermatogenic activity equivalent to Stage IV. One of the latter animals, that had grown more than the rest, had hypertrophied accessory sex organs (Stage III), the other had atrophic accessory sex organs and a spermatic granuloma of the epididymis. The remaining two animals were in spermatogenic Stage VI. The epididymis and ductus deferens were either empty or contained cellular debris. None of the accessory sex organs was secretory (Stage V). Four adult lizards on 9L from January 9 to April 4 were in spermatogenic Stage IV and the accessory sex organs approached Stage III. Similarly, ten adults on 9L from February 3 to April 4 were in spermatogenic Stages III and IV and their accessory sex organs in Stage III.

Experiment 7. April–June: Adult lizards exposed to 6L and natural day-length

On April 30, 26 adult male lizards were placed on a regime of 6L. Five of these animals were sacrificed on June 3 and compared to a series of 24 control animals maintained on natural day-length. Testis weight was very significantly less in the 6L sample (22 to 33 mg; 29.5 ± 3.1 mg.)[1] than in the controls (24 to 52 mg.; 37.0 ± 6.5 mg.), but was larger than that of two samples of wild animals taken in June (Fig. 2). Spermatogenesis in the 6L animals was in late Stage IV typical of wild animals in July, whereas the controls were in spermatogenic Stages III and early IV. The accessory sex organs of the 6L sample were secretory and were not significantly different from those of the controls.

Nine additional animals from the 6L sample were sacrificed on June 21 and compared with a sample of nine newly captured animals. Testis weights were very significantly less (4 to 22 mg.; 14.9 ± 5.5 mg.) than those of wild animals (16 to 33 mg.; 23.7 ± 5.6 mg.). Eight of the 6L animals were in spermatogenic Stage V (typical of August animals) and one in Stage VI. All wild animals were in spermatogenic Stage IV. The epididymis and ductus deferens were significantly reduced in size and in secretory activity in a few experimental animals. All experimental animals showed significant reduction in epithelial height of the sexual segment, but a few retained secretory activity in the distal end of the tubules.

An attempt was made to determine whether the precocious termination of the sexual cycle, which was brought about by exposure to short days, resulted in a temporary gonadal refractoriness to stimulation by long day-lengths. Six animals which had been maintained on 6L from April 30 to June 20, were exposed to 18L from June 21 to September 18. Three responded markedly and three did not. Testis weights (3 to 17 mg.; 10.8 ± 7.2 mg.) were significantly heavier than those of a sample of eight controls maintained on natural day-length from June 4 to

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[1] Range; mean and standard deviation.

September 18 (2 to 6 mg.; 3.5 ± 0.6 mg.) and a sample of nine wild animals killed September 22 (1.5 to 4 mg.; 2.3 ± 0.7 mg.). Controls and wild animals were at the end of the annual spermatogenic cycle (Stage VI) and all accessory sex organs were atrophic. Of the six experimental animals two were in spermatogenic Stage VI, one advanced to Stage I, and three advanced to Stage II. The latter three individuals exhibited a precocious spermiogenesis and the epididymis was hypertrophied and contained sperm. The ductus deferens and sexual segment were secretory (Stage II) in only a single specimen in which sperm had reached the ductus deferens. The two unresponsive animals were seriously starved.

Experiment 8. May–July: Adult lizards exposed to 18L, natural day-length and 9L

At the initiation of this experiment the reproductive system of newly captured animals was near peak development (Stage III). During July wild lizards undergo a regression of spermatogenesis (Stage IV), but the accessory sex organs are still near maximum development. The two experimental samples for this period had a relatively high mortality due to starvation (Table II). Spermatogenesis was exhausted (Stage VI) in the 9L sample (Fig. 1) and the accessory sex organs were atrophic (Table II). Considerable variation existed in the 18L sample. Its average testis weight (Fig. 1) was very significantly less than that of controls exposed to natural daylight, but it was nearly equal to the average for wild animals in July. Only one individual was markedly stimulated by the excessive light treatment. Its reproductive organs were at peak activity and comparable to those of an animal in April or May. Except for a slight increase in the number of primary spermatocytes, the gonads of three animals were comparable to those of control or wild animals in July; the sexual segment of the kidney, however, was less hypertrophied. The two remaining lizards had smaller gonads and an atypical spermatogenic pattern. The germinal elements closest to the lumina of the seminiferous tubules were all advanced spermatids (Stage V) whereas the outer layers revealed a proliferation of spermatogonia and primary spermatocytes (Stage I). In one of these animals the ductus deferens and ductus epididymidis were moderately hypertrophied and contained sperm, in the other these organs were atrophic. The sexual segment was inactive in both. Since these animals gained weight, the poor response to light did not appear to be due to starvation.

Experiment 9. July–September: Adult lizards exposed to 18L and natural day-length

The sample means of the experimental animals were very significantly greater for all measurements taken (Fig. 1 and Table II). The testes of the controls were lacking in spermatogenic activity (Stage VI). The testes of the experimentals varied from 5 to 34 mg. and were judged to be in spermatogenic Stages I–IV.

Control and wild animals sacrificed in September have atrophic accessory sex organs. Among animals maintained on 18L, the ductus epididymidis and ductus deferens were highly convoluted, filled with sperm and secretory in twelve; nearly empty and much reduced in three; empty and atrophic in two. The sexual segment of the kidney was hypertrophied and secretory in the ureter and distal ends of the ducts in six, and non-secretory in the others. The animals that were least responsive to the light treatment possessed fat bodies weighing around 100 mg. (5% of body weight). This represented half or less the weight of fat bodies of animals that yielded a good response.

Experiment 10. August–October: Adult lizards exposed to 18L, 14L, and natural day-length

This experiment was initiated at a time when rapid involution was occurring in all reproductive organs. By the end of the experiment both wild animals and controls had completed a reorganization of these organs and were ready to initiate a new cycle. The testes of most animals in both experimental groups were markedly ($P < 1\%$) enlarged (Fig. 1). Both the maximum and average responses of the 14L sample were considerably less than those of the 18L sample in respect to testis weight and spermatogenic development (Table II). On the basis of a highly convoluted and sperm-packed ductus deferens, four lizards on 18L were classified in spermatogenic Stage IV. The fat bodies of the unresponsive lizards on 18L weighed half as much as those that responded well. Most of the unresponsive animals on 14L had very large fat bodies.

Hypertrophy of the accessory sex organs of the 18L sample tested to be very highly significant except for the sexual segment of the kidney for which $P = .02$. Only the four animals classified in spermatogenic Stage IV showed secretion in the middle and distal portions of the collecting ducts. The hypertrophy of the accessory sex organs of the 14L sample proved to be significant, although no secretion was found in the sexual segment of the kidney.

DISCUSSION

Degree of response of Anolis to artificially lengthened days. In the fall, when gonads are smallest, a 60-day exposure to 18L was sufficient to produce numerous spermatozoa in the larger testes and a precocious, but limited, spermiogenesis in the smaller ones. Although the size of the gonads was only about half that of animals during the breeding season, the relative increases were the greatest of the entire series. Assuming that testes weights were equivalent to those of wild anoles at the beginning of the experiments, gonadal weights of both adults and immatures underwent at least a six-fold increase (Experiments 1 and 2). The closer to the breeding period (April through August) an experiment was started, the more complete was gonadal response. The relative increase in weight, however, was proportionately less.

A similar seasonal gradient in responsiveness was observed in the accessory sex organs. The sexual segment is a more reliable index of this gradient than are the epididymis and vas deferens in which the apparent activity varies with sperm content. Exposure to 18L for 60 days starting in September or October (Experiments 1 to 3) did not bring the sexual segment of adult or immature lizards into a secretory condition typical of the breeding period. However, in Experiment 4, started November 14, half of the adults developed secretory sexual segments typical of April breeding animals. Progressively greater responses were obtained in each succeeding experiment through the winter (Table II).

Our data on the completeness of the testicular response correspond reasonably well with the results of others working with reptiles and birds (for references see reviews by Hammond, 1954 and Farner, 1955). Only rarely do experimental, photoperiodic-stimulated gonads achieve the size that is typical of the particular species during the breeding season; further, the largest gonads are invariably induced just prior to the natural time for hypertrophy. The data of Vaugien (1955) on testis size and bill color of immature English sparrows best illustrate the seasonal gradient of response.

Several factors have been suggested to account for the seasonal variation in the response of the reproductive system. Vaugien (1955) considered the increasing responses which he observed to be due, in part, to the increasing age of the immature sparrows. In immature anoles the degree of response appears to be correlated with growth rate as well as age (as determined by size). Vaugien also demonstrated that in immature English sparrows the longer the exposure to short days prior to capture, the greater the testicular response to long day-lengths. Exposure to a period of short days in the fall is apparently essential for spermatogenesis in a number of species of birds. In adult anoles, exposure to long day-lengths during or following the involution of the testes (Experiments 9, 10, and 1) elicited a response without prior exposure to short photoperiods.

It is generally accepted that the annual variation of the reproductive systems of wild vertebrates is primarily determined by the cyclic nature of pituitary secretions. However, Van Oordt (1956) demonstrated a seasonal difference in the sensitivity of spermatogonia to pituitary gonadotropins in the frog, *Rana temporaria*. On the basis of thyroxin injections, Vaugien (1955) postulated that during short days the testes of immature English sparrows become increasingly susceptible to stimulation. It is possible, however, to explain adequately the data on *Anolis* without assuming a seasonal change in sensitivity on the part of the reproductive system. The gradient of response of the testes can be accounted for by the number of spermatogonia present at the beginning of each experiment, there being fewest in September and progressively more during the winter. The gradient observed in the accessory sex organs appears to depend both upon the state of their respective cells and the state of the interstitial cells at the beginning of each experiment. In September the reproductive organs are atrophic and secretory interstital cells are virtually absent (Fox, 1958). At this time, a long period of stimulus appears necessary since the interstitial cells must be brought into activity before their secretions can secondarily stimulate the accessory sex organs. During later months hypertrophied interstitial cells are increasingly more abundant. These probably immediately release androgenic hormone which, in turn, would bring about progressively greater enlargements of the already partially hypertrophied accessory sex organs.

The above explanation could account for the marked response of some individuals in Experiments 9 and 10. Although the reproductive organs and interstitial cells of these animals were declining at the start of the experiments, they were not atrophic and could respond rapidly to a new stimulus. Conversely, the results of Experiment 7, in which lizards hastened into an early atrophy of the reproductive organs by exposure to 6L responded only mildly to exposure to 18L, could be explained by the degree of atrophy at the time the long day stimulus was applied.

On the basis of data obtained by exposing normal and pinealectomized anoles to normal and long days, Clausen and Poris (1937) have suggested that the pineal eye inhibits the testicular cycle. It is interesting to compare data from our Experiment 4 with their data since the two experiments were conducted at approximately the same time of the year. The testes weights of our animals on either long or short days during this period appear to be nearly identical to those of their pinealectomized animals on similar light regimes. Of the two samples they maintained on normal day-lengths the unoperated controls averaged 0.8 g. less in weight at the beginning of the experiment. The average weight of this sample (3.92 g.) suggests that it was composed largely of sub-adult animals. Since testis weight in *Anolis* has been shown to regress significantly with body size (Fox, 1958), the differences between the two samples could be due to inequalities in sampling. Similarly, in their two samples kept on a long day program, the unoperated animals averaged 0.7 g. lighter than the operated. However, even without this consideration it is doubtful whether the minor difference of 1.5 mg. between the mean testis weights would prove to be statistically significant. In our opinion, the data of Clausen and Poris (1937) do not afford adequate proof that the pineal eye acts as an inhibitor of the male reproductive system in *Anolis*.

Studies on *Anolis* and other species emphasize the importance of examining reproductive structures other than the testes or sperm-storing organs when determining the breeding status of an animal. The presence of viable sperm may not always be a reliable indicator as to whether or not an individual is in full breeding condition. Experiments 1, 2, and 3 indicate that the response of the accessory sex organs may lag behind spermatogenesis. Further, in many species the male normally stores sperm during a non-breeding stage of the reproductive cycle (Fox, 1952; Harrington, 1956). To determine the true breeding status some reliable test for the presence of androgenic secretions should accompany tests for viable sperm. In the English sparrow the color of the bill serves this purpose (Keck, 1933). In many species the emergence of characteristic behavioral patterns or, more directly, the histology of the interstitial cells has been correlated with androgenic activity. We have made only cursory observations on the above criteria in this study. The nuchal crest, a secondary sex character, appeared to enlarge in animals brought into full breeding condition. Although no detailed records of behavior were made, we observed frequent attempted copulations between males exposed to long days in the winter and spring experiments.

The histology of the sexual segment of the kidney in lizards can be used as an accurate measure of androgenic activity and the stage of the breeding cycle. First, cell height and tubule diameter can be measured with precision. Second, the progressive spread of secretion granules can be traced both intracellularly (from the apices to the basal nuclei of the tall columnar cells) and from the ureter and distal ends of the collecting ducts to the proximal collecting ducts.

If the presence of viable sperm alone were used as an indicator it would appear that male anoles were brought into breeding condition by exposure to 18L for 60 days at any season of the year. However, examination of the sexual segment reveals that none were brought into breeding condition in experiments started in September and October and only half of those in experiments started in November.

Refractoriness to photoperiodic stimulation. Passerine birds characteristically exhibit a refractoriness to stimulus by long photoperiods at the close of the breeding season. This refractoriness persists until there has been a period of exposure to long nights (Wolfson, 1952). The refractory period allows for the physiological reorganization of the gonadal and fat cycles (Wolfson, 1954) and is probably caused by seasonal reduction in pituitary activity (Miller, 1948, 1949; Farner and Mewaldt, 1955).

We have been unable to find a period during which *Anolis* is completely refractory to photoperiodic stimulation. The testes seem to respond, to some extent, at all seasons of the year (Fig. 1, Table II). In all experiments except Nos. 5 and 6, which were started near the peak of testicular development (Stage III), a longer photoperiod resulted in either greater mean or maximum testis weights. In Experiments 5 and 6, although there was no quantitative increase, there was a premature progression to a more advanced stage of spermatogenesis. Experiments 8, 9, and 10, initiated after the peak of testicular size had been achieved, yielded variable results. Normally, animals at this time should have progressed from spermatogenic Stages III or IV towards Stages V or VI with the number of dividing spermatogonia and spermatocytes rapidly decreasing. In some individuals, however, all classes of germinal cells were well represented so that the gonad appeared to be in Stage III. In other individuals spermatogenesis was maintained at about a Stage IV level without showing the expected decline. In still others, germinal elements characteristic of Stages I and V were present in the same seminiferous tubule, although intervening stages were missing. This suggests a premature beginning of a new cycle before the completion of the old.

The sexual segment of the kidney appeared to be refractive in experiments started in September and only mildly responsive in October. Although this could be due to a difference in the cyclic nature of the two pituitary gonadotropins as suggested by Farner and Mewaldt (1955), we believe that the poor response of the sexual segment can be accounted for by the delay in arousing the atrophic interstitial cells.

Inherent rhythm. In studying the natural reproductive cycle of male anoles, Fox (1958) noted that spermatogenesis is initiated in the fall and makes considerable progress during the winter despite the short day-lengths. We sought to determine if there was any period during the year in which constant exposure to short days would disrupt the normal cycle. Nine hours of artificial light were chosen instead of 10 (day-length at time of the winter solstice at New Orleans) since 10 hours of bright light obviously are not available during the winter. Nine hours did not disrupt the cycle of immature lizards exposed from October through December and from January through March, nor that of adults exposed from December through February. The accessory sex organs likewise were not retarded by the short days. In fact, in adults of the 9L sample killed in February, both the testes and accessory sex organs were more advanced than those of wild lizards at that date. At the time of writing, additional data were furnished us by Anthony Dimaggio of our Biochemistry Department. Six immature males (49–57 mm.), maintained at 6L for 60 days ending March 1, appeared to have normally active testes (left testis ranged 12–40 mg., mean = 24.3 ± 11.4 mg.). One must conclude that reasonably short day-lengths are not very effective in disrupting the inherent reproductive rhythm of male anoles in the fall, winter and early spring when gametogenesis is on the upswing.

Fox (1958) also stated that the peak of spermatogenesis for most individuals of this species was achieved in April. Normally, all maintained quite active spermatogenesis through July. Experiment 7 showed that six-hour day-lengths, initiated at the end of April, produced a significant reduction in testis weight within 34 days, and highly significant atrophy in both the testes and accessory sex organs within 52 days. Nine hours of light (Experiment 8) from May to July also precipitated complete involution. Thus, it appears that short days will end a reproductive cycle prematurely after the cycle has neared or passed maximum development.

The stimulating effects of different day-lengths. Since our original choice of 18 hours for a long day stimulus was arbitrary, we designed three experiments to test whether shorter periods might be equally stimulating but have fewer detrimental effects upon the animals. In Experiment 3 all average measurements of the 16L sample tended to be greater than the 18L sample. However, the larger gonads and more secretory sexual segments occurred in the 18L sample. In Experiment 5 a few adults on 16L gave a better response than any on 18L. The smaller gonads of the 18L sample were judged on a histological basis to be more advanced than those of the controls, but there does not appear to be justification to similarly appraise the smaller accessory sex organs. We believe the data for the 18L sample reflect the exhausting features of long hours of wakefulness imposed upon animals with initially low fat reserves, rather than a lack of stimulation. Experiment 10 demonstrated that exposure to 14L from August to October was stimulating (Fig. 1) to the testes but had little effect on the accessory sex organs. On the other hand, 18L was very stimulating to both gonads and accessory sex organs.

These data indicate that, in general, the longer the day-length the greater the response of the reproductive organs of *Anolis*. A similar relationship between day-length and gonadal size of the white-crowned sparrow has been thoroughly analyzed recently by Farner and Wilson (1957). Eighteen-hour day-lengths appeared detrimental to many anoles (see mortalities and starvation, Table II). For this reason a 16-hour day-length is probably more satisfactory to use as a long day stimulus for *Anolis*. Fourteen-hour day-lengths (the maximum day-length for New Orleans) are definitely stimulatory to *Anolis*, but the response may not be sufficiently rapid to be detected in short-term experiments. Dessauer (1953) found no differences in the metabolism of anoles exposed for three weeks to 10L and 14L. It now seems likely, in view of the considerable individual variation he obtained, that three weeks may not have been sufficient or the light regimes not sufficiently different to produce marked contrasts in metabolism.

The response of immature lizards compared to adults. The usefulness of immature anoles as experimental animals was impaired by their high mortality rate which resulted from starvation. However, since they were more abundant than adults in the wild it was easier to collect a large series. They responded to the long day-length exposure very much as did the adults without any indication of a refractory period. Comparison of the September experiments (I and II) indicates that the non-starving immature lizards responded as well as or better than the adults. Likewise, the maximal measurements recorded in Experiment 6 (Table II, Fig. 1) compare favorably with the best results on adults in any experiment. The most striking responses occurred in animals above 55 mm. (snout-vent

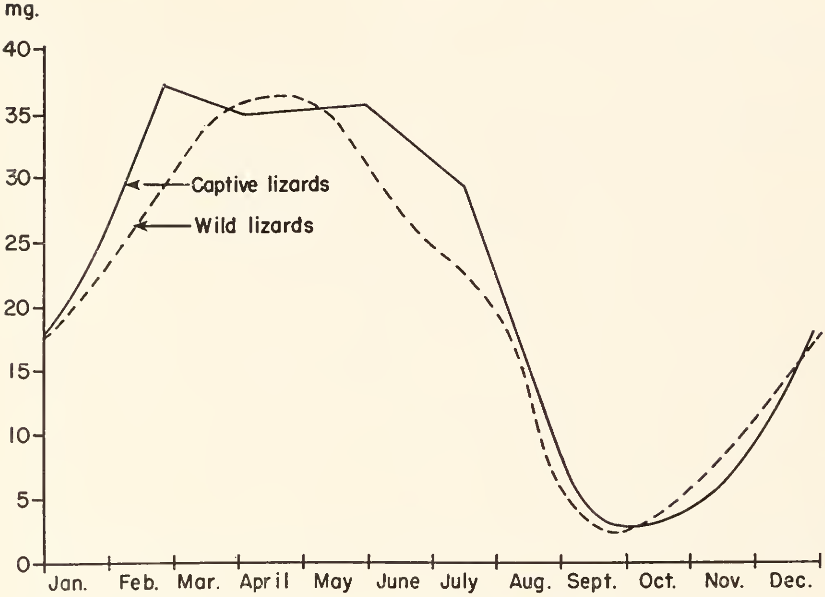

FIGURE 2. Curves comparing the annual variation in testis weights of freshly captured wild anoles (Fox, 1958) with those of laboratory controls exposed to natural day-lengths for 60 days.

length). All individuals with markedly active reproductive organs grew at least several millimeters and usually reached 60 mm. in snout-vent length.

Data on animals maintained for periods longer than two months suggest that unseasonal long photoperiods are most stimulating during the first two months. Over a period of four months the controls matched the earlier achievements of the experimentals and the experimentals regressed.

Captive lizards exposed to natural day-lengths compared to wild lizards. The marked weight difference between testes of two-month captive lizards exposed to natural day-lengths and wild lizards can be seen in Figure 2. From February through most of July the testes of captive controls usually averaged significantly heavier than those of wild animals sacrificed at approximately the same date. Bartholomew (1950) found similar differences in captive and wild yucca night lizards (*Xantusia vigilis*). He suggested that the differences were due to the higher temperature of the laboratory. In the case of *Anolis*, this could easily account for the differences during the winter and spring but it is doubtful whether it would account for the differences in June and July.

SUMMARY

1. The reproductive system of adult male anoles was stimulated by artificially lengthened days at all seasons of the year. At least a few males were brought into full breeding condition in experiments initiated from November through August. Sperm were produced at all seasons.

2. A refractory period, such as occurs in passerine birds, did not appear to be present in *Anolis*.

3. A previous exposure to short days was not essential for obtaining a response to a long photoperiod.

4. Both the testes and accessory sex organs exhibited progressively greater responses in successive experiments through the fall, winter, and spring.

5. The seasonal gradient of response which produced increasingly larger testes could be accounted for by the increasingly larger number of spermatogonia at the beginning of each experiment. The gradient in response of the accessory sex organs was dependent on the normal cyclic fluctuations of their histology and the abundance of hypertrophied interstitial cells.

6. The inherent rhythms of both the gonad and accessory sex organs were not disturbed by 60 days' exposure to 9- or 6-hour photoperiods during the fall and winter. However, such exposures near or after the peak of spermatogenesis resulted in premature atrophy of all reproductive organs.

7. Comparison of the effects of 14-, 16-, and 18-hour day-lengths suggested that the longer the day-length the more rapid the response. Extremely long days, however, appeared to have detrimental effects upon some individuals.

8. Most immature lizards of less than 55 mm. snout-vent length responded poorly to the experimental conditions. All that responded well were growing rapidly. Those that grew to 60 mm. or more had reproductive organs as large or larger than older adults.

9. Captive lizards exposed to natural day-length for 60 days at any time between February and July tended to have larger testes than wild lizards sacrificed on the same date.

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A PERSISTENT DIURNAL RHYTHM OF LUMINESCENCE IN GONYAULAX POLYEDRA[1]

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The photosynthetic marine dinoflagellate, *Gonyaulax polyedra*, emits a brief flash of light (duration, about 90 milliseconds) when stimulated by agitation. It is one of the many organisms responsible for the luminescent display sometimes observed in the ocean at night when the water is disturbed (see Harvey, 1952). Previous studies with this organism (Haxo and Sweeney, 1955; Sweeney and Hastings, 1957a) have shown that the luminescent response to stimulation varies rhythmically in a diurnal fashion. Cultures grown in natural illumination, or in artificial lights with alternating light and dark periods of 12 hours each (= LD), display a much greater luminescence during the dark period (Fig. 2).

When LD cultures are transferred to a dark chamber, the rhythm continues but its amplitude decreases progressively. By action spectra studies, it has been found (Sweeney, Haxo and Hastings, unpublished data) that this decrease in amplitude arises from the need for light in the organic nutrition of *Gonyaulax*, via photosynthesis. This finding prompted the search for constant environmental conditions under which the endogenous rhythm would persist, without the loss of amplitude which occurs in continuous darkness.

The possibility of maintaining the cells heterotrophically was explored, but the consistently negative results obtained indicated that *Gonyaulax* is an obligate photo-auxotroph. Continuous bright light inhibits the rhythmic fluctuations in luminescence, and it has not been possible to separate, by using light of different colors, the photosynthetic requirements for light from the inhibitory action of light on rhythmicity. It has been found, however, that if LD cultures are placed in a continuous dim light, the rhythm of luminescence persists without loss of amplitude. It has thus been possible to investigate in some detail the nature of this endogenous rhythm.

MATERIALS AND METHODS

G. polyedra has been maintained in a modified sea water medium described previously (Sweeney and Hastings, 1957a). The growth rate is dependent upon light, temperature, and the concentrations of mineral nutrients. The maximum growth rate which we have measured is one division per day, but under the condi-

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[1] This research has been supported in part by grants from The National Science Foundation, the Graduate Schools of Northwestern University and the University of Illinois, and the Marine Life Research Program of the California Cooperative Oceanic Fisheries Investigation. Part of the research was carried out at Northwestern University, Evanston, Illinois. Contribution from the Scripps Institution of Oceanography, new series.

tions used in the experiments to be described the rates were always less than this. The illumination was provided by "cool white" fluorescent lamps, the intensity being measured in foot-candles with a Weston illumination meter.

The experimental procedure was as follows: stock cultures were maintained in Fernbach flasks containing 1500 ml. of medium. In preparation for an experiment, 2-ml. aliquots from these cultures were pipetted into each of several hundred test tubes at cell densities between 2000 and 7500 cells per ml. All tubes were then subjected to the appropriate conditions of light and temperature. To measure the luminescence at any given time, two tubes were removed, assayed, and then discarded. The cells were stimulated to luminesce by bubbling air through the cell suspension, and the resulting phototube current was accumulated on a capacitor. Luminescence is expressed in terms of the total amount of light emitted during one minute of stimulation, at the end of which time essentially all luminescence has ceased. Additional details of the light measurement procedure may be found elsewhere (Sweeney and Hastings, 1957a).

RESULTS

Demonstration of the persistent rhythm. A persistent rhythm of luminescence may be observed if cells which have been kept for a time under LD conditions are transferred to continuous dim light (about 100 foot-candles). A typical example of the persistent rhythm under conditions of constant light and constant temperature is shown in Figure 5. In similar experiments, we have continued measurements for as long as 14 days; the rhythmic pattern continues undamped during this time. At the light intensity used in such experiments there was little growth.

The natural period of the rhythm. The period of the rhythm is measured by the time between successive maxima in luminescence. When the cells are subjected to alternating light and dark periods on a daily (24-hour) schedule, the period of the rhythm is 24 hours (Fig. 2). Under conditions of constant illumination, however, the rhythmic changes have a period which is close to, but not necessarily exactly 24 hours. Pittendrigh and Bruce (1957) have referred to this as the *natural period,* or the innate period of an endogenous rhythm when light and temperature are held constant.

The natural period in *Gonyaulax* is a function of at least two environmental factors, light intensity and temperature. The effect of light intensity upon the period is illustrated in Figure 1. Cells were placed in continuous light at three different intensities, and it is evident that the natural period was shorter at higher intensities. These experiments also illustrate the light intensity dependence of the inhibitory effect of continuous illumination upon the rhythm. At the two higher light intensities the amplitude of the rhythm was progressively damped, while at the lowest light intensity no marked damping of the amplitude of the rhythm was evident.

The effect of temperature upon the natural period is not large but, contrary to expectation, the period becomes longer rather than shorter as the temperature is raised (Hastings and Sweeney, 1957b). At 16° C. the period was found to be 22.8 hours while at 26.7° C. it was 26.5 hours. A Q_{10} of less than 1.0 is unusual, and the results were interpreted as evidence for a compensation mechanism which functions to keep the period approximately temperature-independent.

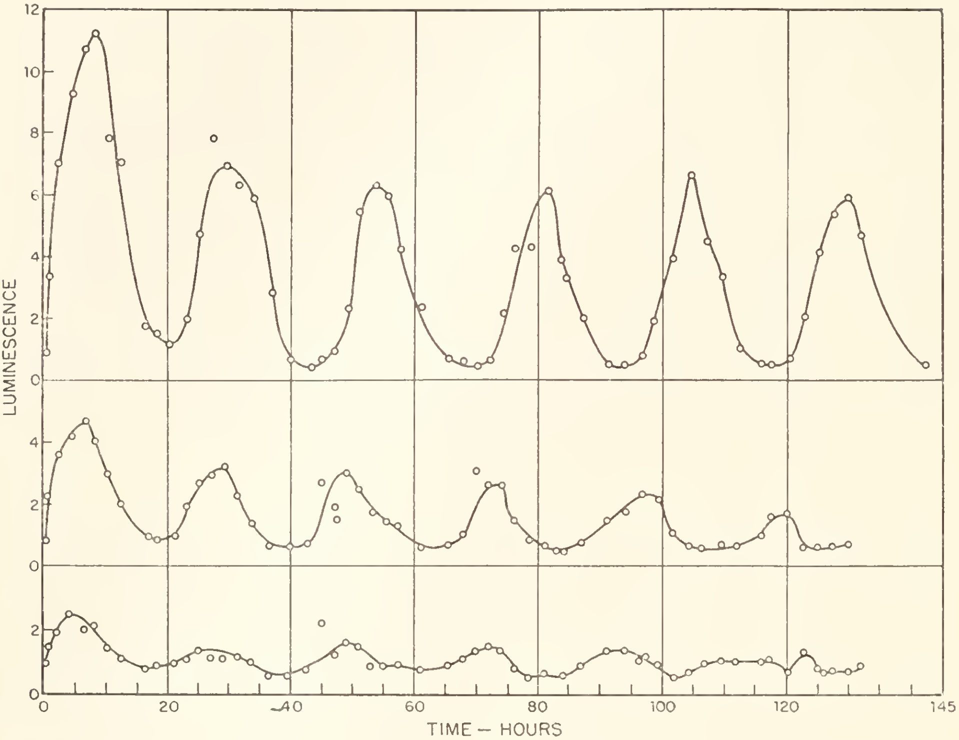

FIGURE 1. The effect of light intensity upon the natural period at constant temperature (21° C.). The cells were grown in LD conditions (800 foot-candles during the light period). The beginning of the experiment, shown on the graph as 0 time, fell at the end of a normal light period. At this time, some cells were placed in the dark, and others in light of 120 foot-candles (upper curve), 380 foot-candles (middle) and 680 foot-candles (bottom). The average periods were as follows: 680 foot-candles, 22.0 hours; 380 foot-candles, 22.8 hours; 120 foot-candles, 24.5 hours; dark, 24.5 hours (not shown on graph; one period measured).

In view of the relatively small temperature effect, the period of this rhythm may be characterized as essentially temperature-independent.

The endogenous nature of the diurnal rhythm. The persistence of the rhythm of luminescence under conditions of constant temperature and light intensity indicates that the mechanism of the rhythmicity is endogenous. Several other experiments serve to support this conclusion.

Figure 2 illustrates one of many experiments in which the phase of the rhythm was shifted by changing the time at which the light and dark periods occurred. In such experiments the phase (*i.e.*, the solar time at which the maximum in luminescence occurs) may be shifted so that it will bear any desired relationship to the solar day. In cultures which are subsequently transferred to constant conditions of dim light or darkness, the phase of the persistent rhythm is related to the previous light and dark program rather than to solar time, or any other factor. Changes in the phase of the endogenous rhythm have not been observed when light and temperatures were held constant.

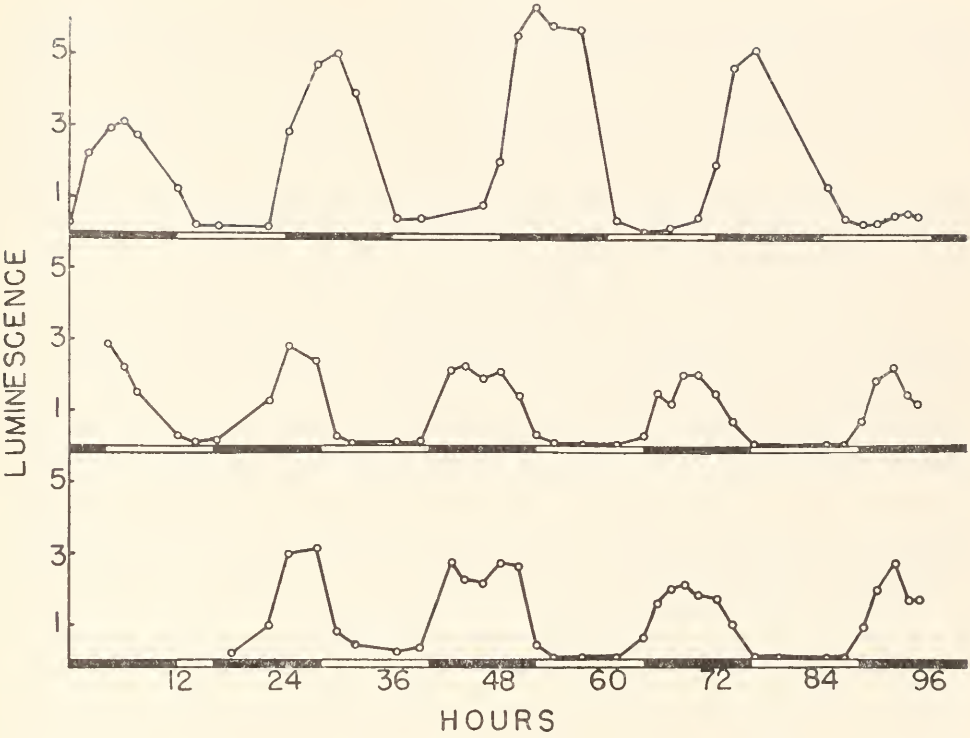

FIGURE 2. This experiment illustrates the effect of changing the solar time at which the light and dark periods occur. The upper curve shows the pattern of luminescence changes in an LD culture which had been on the schedule indicated for some time. The black bars on the time axis indicate dark periods. The lower two graphs illustrate the effect of imposing upon cultures (which were previously on the schedule shown in the top graph) an LD schedule in which the light and dark periods were at a different time of day. The new schedules were started at zero hours on the graph. Temperature, about 26° C. Light intensities used, about 250 foot-candles.

A series of experiments has been carried out from which it is evident that pre-treatment with diurnal light and dark periods (*i.e.,* one dark plus one light period equals 24 hours) is not necessary in order to demonstrate an endogenous rhythm. That is to say, there is no evidence that a "learning" or "memory" process is involved. For example, cells have been exposed to "non-diurnal" light and dark periods which together add up to greater or less than 24 hours, followed by conditions of either constant light or constant dark. An experiment of this sort is shown in Figure 3. In this experiment, cells were exposed to alternating light and dark periods of 7 hours each for about 100 hours. During this period the luminescence changes were quite evidently governed by these light and dark periods so that there was a maximum in luminescence every 14 hours. At the end of this treatment, some cells were placed in constant dim light and others in darkness. In both cases a diurnal rhythm with a period of approximately 24 hours was evident. The 14-hour cycle had not been "learned," even though it had been possible to entrain the luminescence rhythm to the 14-hour cycle. A difference between those placed in darkness and those in dim light was that the amplitude of the rhythm in darkness progressively decreased as a result of the lack of light (see introduction).

Similar experiments have been carried out in which the alternating light and dark periods were 6 hours each, 8 hours each, and 16 hours each, giving cycles of 12, 16 and 32 hours, respectively. The results were similar to those shown in Figure 3. After about 100 hours of such a non-diurnal light-dark cycle the cells were placed in constant dim light and a rhythm of luminescence having a period close to 24 hours was evident.

Another series of experiments has shown that it is not necessary to pre-treat the cells with any sort of alternating light and dark periods in order to demonstrate endogenous diurnal rhythmicity. As mentioned previously, if cells are grown in continuous bright light (*ca.* 800–1500 foot-candles) there is no detectible rhythmicity. Cells maintained in this way for several months, or for as long as several years, have been found to exhibit a diurnal rhythmicity when they are placed in darkness (Haxo and Sweeney, 1955; Sweeney and Hastings, 1957a). The phase of the rhythm which is initiated when the cells are moved from bright light to darkness is independent of the solar time, and related only to the time at which the light-to-dark transition is made.

A similar result was obtained when cells which had been grown in bright light for almost one year were merely transferred to dim light. This experiment is

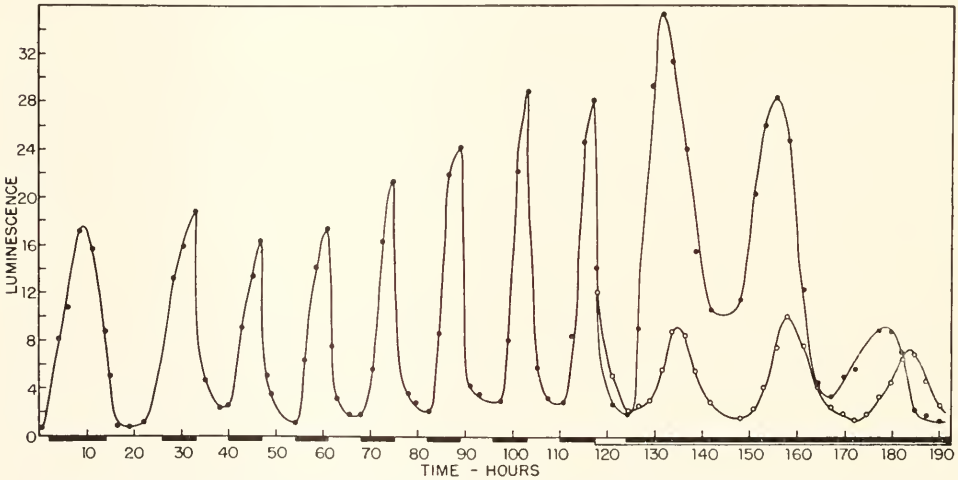

FIGURE 3. This illustrates the entrainment of the luminescence rhythm to a 14-hour cycle and the manifestation of an endogenous diurnal rhythm when the cells are placed in constant conditions subsequent to the treatment. Dark periods are indicated by black bars on the time axis. The cells were on an LD schedule previous to the time when the 14-hour cycle was started (at 26 hours). Light intensity throughout the 14-hour cycling was 800 foot-candles. At 117 hours some aliquots were removed from the dark and placed in constant light at 230 foot-candles. The luminescence changes in these cultures are shown by the circles. From 124 hours on, the other aliquots were left in the dark and the luminescence changes are plotted with solid dots. Temperature, 21° C.

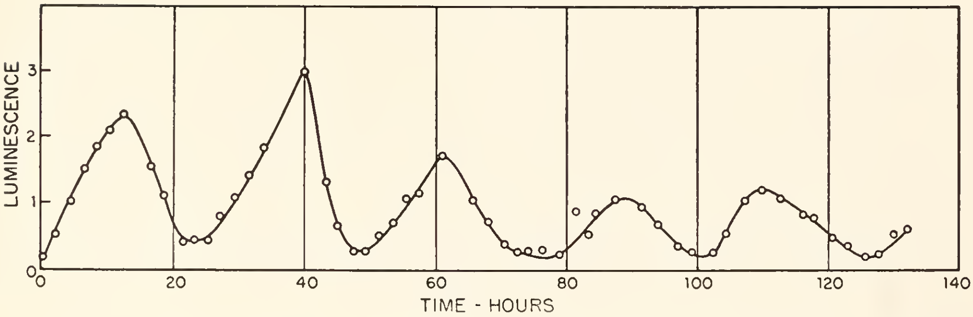

FIGURE 4. The initiation of an endogenous diurnal rhythm of luminescence by means of a one-step change in illumination. Cultures which had been grown in bright light for one year were moved from bright light (800 foot-candles) to dim light (90 foot-candles) at the time indicated on the graph as 0 hours. Luminescence measurements were made approximately every two hours thereafter. Temperature, 21.0° C. Average period, 24.5 hours.

illustrated in Figure 4. It differs from the previously mentioned experiment (in which cultures were moved from bright light to darkness) in that the amplitude does not decrease with time, since light is available for the nutrition of the cells. The precise phase relationship to the time of transfer from bright light is somewhat different, but here also it is not related to solar time.

Phase shift by light perturbation. It is clear from Figure 2 that the phase of the rhythm may readily be shifted by an appropriate manipulation of the light and dark periods to which the cells are exposed. It is not necessary, however, to expose the cells to a new light-dark cycle in order to reset the phase of the rhythm. A single exposure to a different light intensity can result in a stable phase shift. Pittendrigh and Bruce (1957) have discussed the significance of phase resetting of biological rhythms by single light perturbations. If rhythmicity results from an innate oscillatory mechanism characterized by its own natural period, and the phase (but not the period) is determined by the sequence of light and darkness, then it is to be expected that non-repeated light changes should suffice to change the phase. The perturbation therefore need not contain any information concerning period.

The experiment shown in Figure 4 illustrates phase setting by a single step-type light perturbation. The phase of the previously aperiodic cells was determined by the time at which the light intensity was changed. The shifting of phase in already rhythmic cultures is evident in the experiments shown in Figure 3. The entrainment of the rhythm to a 14-hour cycle may be explained by assuming that each transition, either from darkness to light or from light to darkness, serves to shift the phase, so that repetitive phase resetting occurs.

A phase shift in the *Gonyaulax* rhythm by single light perturbations has also been demonstrated in other ways. Figure 5 illustrates a shift in the phase of rhythmic cells which were given a single exposure to either bright light or darkness. The phase shift which results in such experiments has been found to be stable since, in experiments where measurements were continued for an additional 48 hours, the phase difference between the controls and the treated cells remained unchanged.

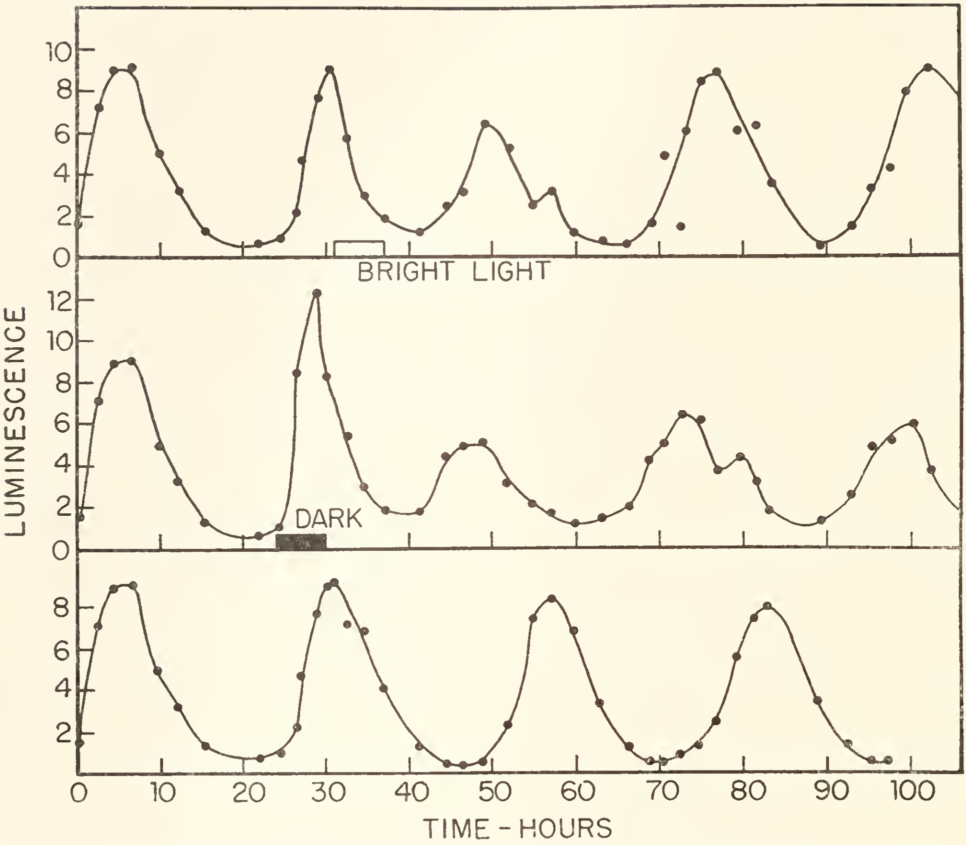

FIGURE 5. This experiment illustrates a phase shift in the rhythm following changes in light intensity. Cells previously kept under LD conditions were placed at constant temperature (23.5° C.) and constant light intensity (100 foot-candles) at the end of a 12-hour dark period. Two days later (zero time on the graph) measurements of luminescence were begun and the endogenous rhythm was apparent. Some cultures (upper curve) were transferred to bright light (1400 foot-candles) for a period of 6 hours and then returned to the previous condition (100 foot-candles). Other cultures (middle curve) were transferred to darkness for 6 hours and returned to dim light at 200 foot-candles. The time at which treatment was given is indicated by bars on the time axis. In both cases a marked phase shift in the rhythm is evident. The control (lower curve) was left in dim light all the while. Average period in control: 25.7 hours.

Figure 6 shows another technique which has been used in the study of phase shifting by single perturbations. Rhythmic cells were placed in the dark and, at a later time, received an exposure to light. Although the amplitude of the rhythm decreases over the next few days, the times at which maxima in luminescence occur are evident, so that the phase may be determined. The number of hours by which the phase is shifted would be expected to be some function of both the magnitude of the perturbation, and the time in the old cycle at which it is administered. The technique of interrupting darkness by light has been used to investigate these parameters.

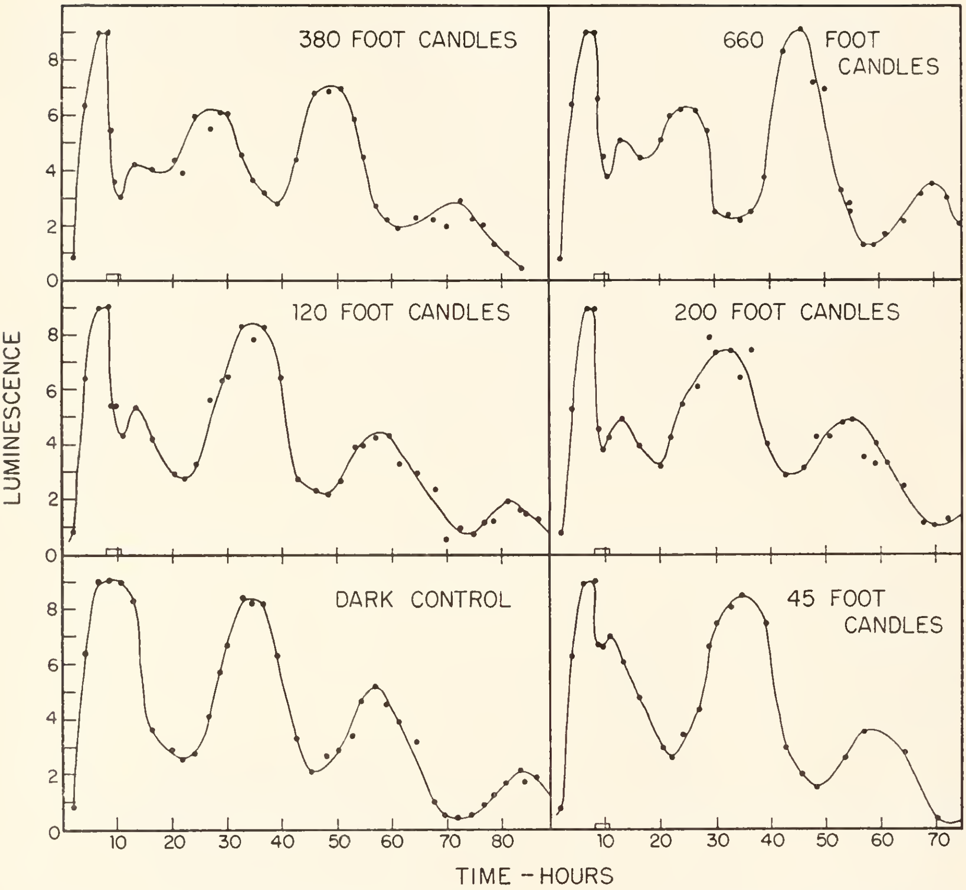

FIGURE 6. This illustrates phase shifting in a rhythmic culture by a single 2½-hour exposure to light, and the effect of intensity upon the magnitude of the phase shift. Prior to the time shown on the graph, all cultures were in LD conditions, and "two hours" on the time axis was the end of the last 12-hour light period. All cultures were put in the dark at that time and the control was left in the dark thereafter. The remaining cells were exposed to a 2½-hour illumination beginning 6 hours after the light-to-dark transition (indicated by the rectangle on the time axis). Following this 2½-hour illumination they were returned to darkness for the remaining time. The intensities used are shown in the figure. A 2½-hour exposure to 1400 foot-candles (not plotted) was found to be no more effective than the exposure to 660 foot-candles (Fig. 7). Temperature during experiment, 21° C.

The effect of varying the light intensity was determined in experiments such as the one shown in Figure 6. The amount of phase shift was found to increase with increasing light intensities, up to a "saturation" value of about 800 foot-candles. This relationship is illustrated in Figure 7, and the stability of the resetting is shown by plotting on the same graph the phase shift measured at each of the subsequent cycles. Several experiments of this sort have been carried out and the same type of relationship has been observed. The quantitative values obtained in separate experiments were somewhat different, however, and the reason for this variation has not been determined.

The magnitude of the perturbation may also be changed by varying the duration of light exposure. In an experiment similar to that shown in Figure 6, the duration instead of the intensity was varied. All exposures (at 800 foot-candles) were started simultaneously, six hours after the cells were placed in darkness. A longer exposure to such a light perturbation was found to be more effective than a shorter exposure. The amount of phase shift was found to be proportional to the duration of the exposure, up to a maximum phase shift of about 11½ hours, which was achieved with 2½ hours exposure. The relationship between phase shift and duration might be expected to be different, depending upon the time in the old cycle at which the perturbations were given, as discussed below. This aspect has not been studied, however.

The effect of varying the time in the cycle at which the perturbation is given has been studied by again using a procedure similar to that used in the experiments shown in Figure 6. Cells grown in LD conditions were transferred to a dark

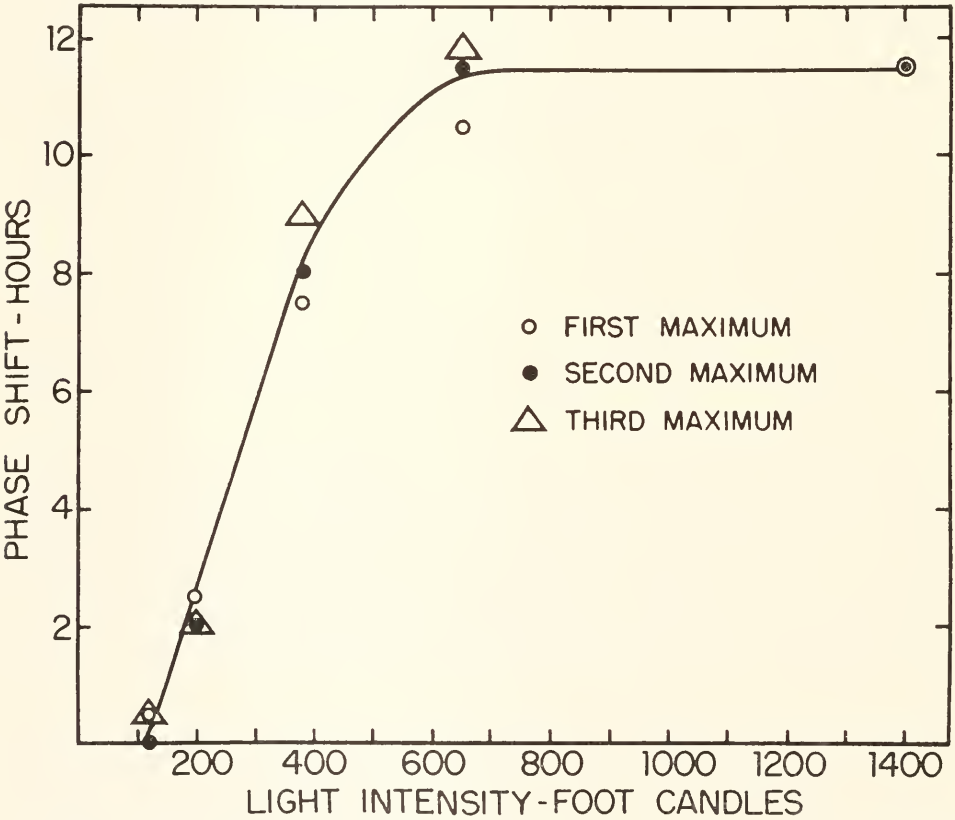

FIGURE 7. The relationship between the intensity of a single 2½-hour light perturbation and the number of hours by which the phase is shifted. Data taken from the experiments shown in Figure 6. Different symbols, as marked on the graph, give the phase difference between the control and the experimentals, measured at each of the three maxima in luminescence subsequent to the perturbation.

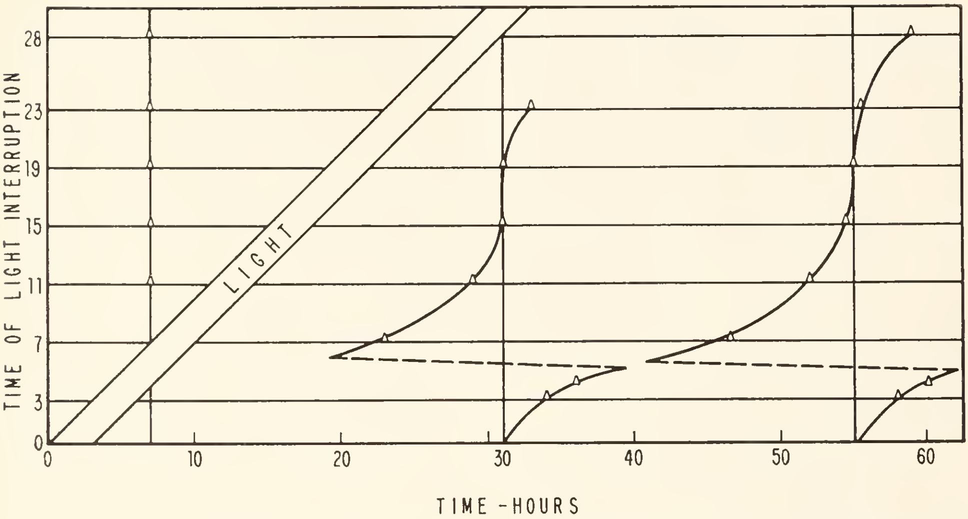

FIGURE 8. The effect of light perturbations (1400 foot-candles for 3 hours) given at different times during the cycle, upon the phase of the endogenous rhythm. Cells which had been kept under LD conditions were placed in the dark at zero time on the graph, which was the end of a 12-hour light period. The times at which the maxima in luminescence occurred in the control, which remained in the dark all the while, are indicated by vertical lines. In the experimentals, a triangular symbol shows a time at which a maximum in luminescence occurred, and thus represents phase. The experiments were carried out in a way similar to those illustrated in Figure 6. Each horizontal line represents a different experiment. For example, the line at 11 hours on the ordinate was an experiment in which a maximum in luminescence occurred at 7 hours. A light perturbation was begun at 11 hours and terminated at 14 hours. Maxima in luminescence occurred subsequently at 29½ hours and 52½ hours. The other experiments are represented in a similar way. The relationship between the time in the cycle at which the light perturbation was administered and the number of hours by which the phase was changed may be better visualized by rotating the figure by 90°.

chamber at the end of a light period. At regular time intervals thereafter, some of the cells were removed and exposed for three hours to light at an intensity of 1400 foot-candles, and then returned to darkness. Times at which exposures to light were made were selected so that the experiment served to scan somewhat more than a full 24-hour cycle. A control received no exposure to light, and the times at which maxima in luminescence occurred in this control are indicated by the vertical lines in Figure 8.

The results of the experiments are summarized in Figure 8. First of all, it may be noted that the new phase, following a light perturbation, was not directly related to the time at which the light perturbation was administered. That is, the maxima in luminescence did not occur at a fixed time interval following the light treatment. If that had been the case, the symbols indicating phase would fall along a line at 45°, parallel to the lines representing the times at which light exposures occurred. This latter type of result was obtained in experiments mentioned previously (Figure 4, for example) where a rhythm was initiated in an arrhythmic culture, and the phase was determined only by the time at which the light intensity was changed.

Secondly, it is apparent that the sensitivity to light perturbations was greater during the first 12 hours (Fig. 8) than during the second 12 hours of the cycle. During the first 12 hours a rather pronounced phase shift resulted, whereas during the second 12 hours there was little or no phase shift. In other longer term experiments it has been found that this variation in sensitivity continues in a rhythmic way. It may therefore be stated that, in general, the cells are maximally sensitive to a light perturbation at a time when luminescence is near maximum, and that this sensitivity declines to a minimum at a time when luminescence is minimum.

Finally, however, it may be noted from Figure 8 that a light exposure given before the maximum in luminescence results in a phase delay, so that the time between the light perturbation and the subsequent maximum in luminescence is greater than 24 hours. On the other hand, a light exposure given after the maximum in luminescence results in a phase advance, such that the next maximum in luminescence occurs in less than 24 hours. This difference is illustrated by the light perturbations which start at three hours and at seven hours in Figure 8.

Perturbation by mechanical stimulation. It is of interest to consider the nature of the cellular component or components which, being modified as a result of the light perturbation, result in the observed phase shift. If perturbation by means other than light also resulted in a change in the components of the rhythmic mechanism, then a phase shift would be similarly expected. It seemed possible that mechanical stimulation might be effective in this regard. Consequently, a perturbation experiment was carried out, in which air was bubbled through the cell suspensions instead of exposing the cells to light (Fig. 9). No phase shift occurred; the cells which had been stimulated retained the same phase as the unstimulated controls.

The experiment also shows that it is possible to modify the concentrations of compounds which are involved in the luminescence rhythm without having any effect upon the phase of the rhythmic mechanism itself. It was found previously (Hastings and Sweeney, 1957a) that the rhythm of luminescence involves a daily variation in the amount of extractable components of the luminescent system (luciferin and luciferase). Mechanical stimulation causes the luminescent reaction to occur, so that one would suppose that the concentrations of components in the luminescent system (and other biochemical systems coupled to it) might be changed. In fact, the apparent effect of stimulation is similar to the effect of light; the luminescence decreases to a low level in both cases. But since no phase shift occurred following stimulation, it does not seem likely that the luminescent system could be directly involved in the basic rhythmic mechanism, although it is clearly coupled to such a mechanism. Moreover, it is evident that there is no feedback from the luminescent system to the system controlling the phase of the rhythm. From previous evidence we had suggested that the luminescent system might itself constitute an autonomous chemical oscillation (Hastings and Sweeney, 1957b). The results described above, however, favor a hypothesis which proposes a basic mechanism of cellular rhythmicity to which various physiological and biochemical processes, such as luminescence or cell division (Sweeney and Hastings, 1957b; 1958), could be coupled.

Cellular interaction. Since all the experiments which have been described are carried out with large cell populations (4000–15,000 cells per tube), the ques-

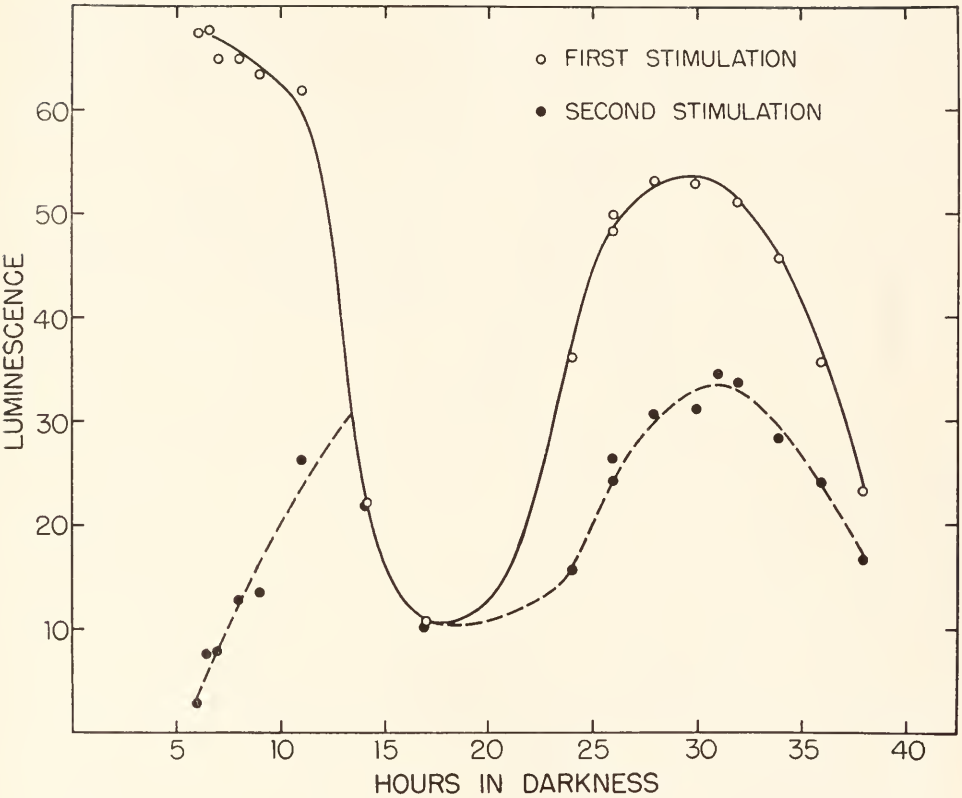

FIGURE 9. The effect of perturbation by mechanical stimulation upon the phase of the rhythm. Previous to the time shown, all cultures were in LD conditions, and zero time on the graph was the end of a light period. At this time all aliquots were placed in the dark. Six hours later a large number of aliquots were stimulated by bubbling air, but were not exposed to light. The luminescence changes of both these and the controls were determined through the subsequent maximum in luminescence. No significant change in the phase of the stimulated cultures was observed.

tion arises as to whether or not some cellular interaction might occur. Since the rhythmic mechanism involves fluctuations in the concentrations of chemical components within the cells, it is conceivable that certain diffusible compounds might escape into the medium, and that their concentrations might also fluctuate in a diurnal fashion. The importance of such a phenomenon would be evident if the supposed compound or compounds could function, as in a feedback mechanism, for stabilizing the frequency and/or phase of the rhythm. It is also possible that some other phenomenon, such as cellular motility, could be involved in such a feedback mechanism. This latter possibility seems unlikely, however, in view of the fact that mechanical stimulation, with its attendant violent motion and disturbance of cellular motility, did not result in a phase change.

An experiment in which this question was investigated is illustrated in Figure 10. Two cultures were maintained under LD conditions for several weeks with their phases different by 5 hours. Samples were pipetted from each culture and moved to constant dim light at the end of a dark period. After each had been under constant conditions for several days (their phases still being different by

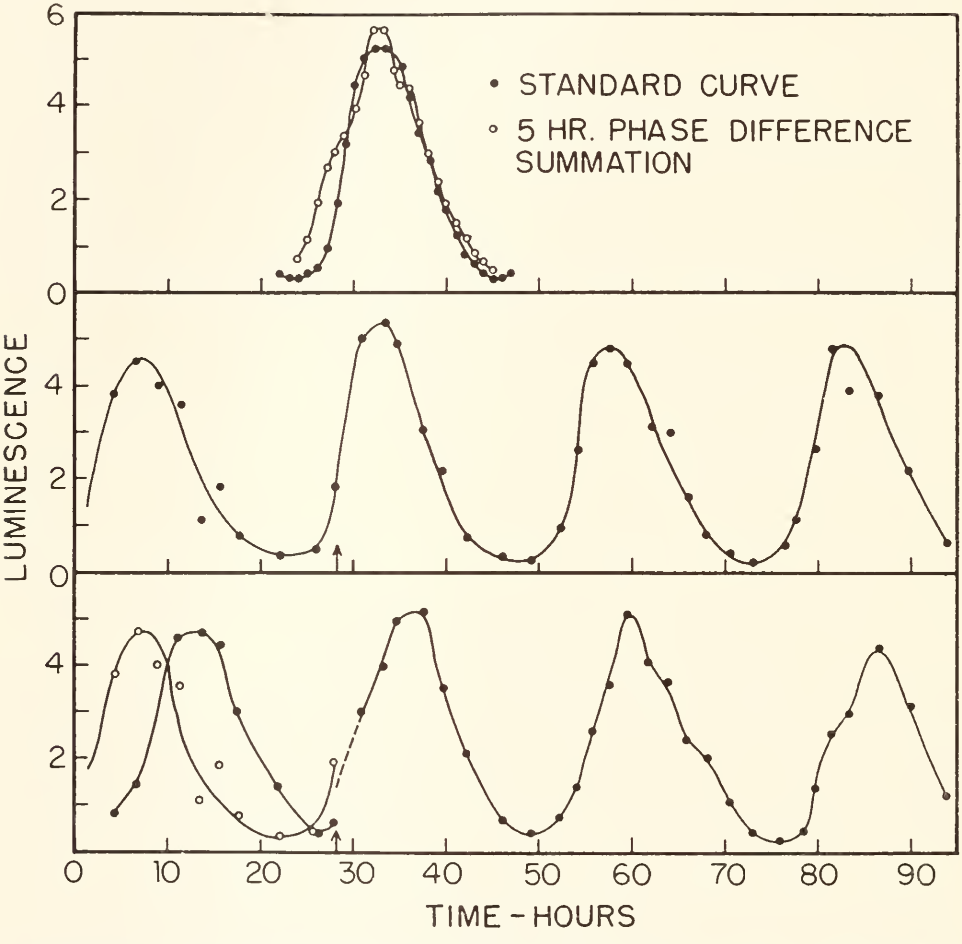

FIGURE 10. The effect of mixing two rhythmic cultures which were out of phase with one another. Cultures which had been in constant dim light for several days, having a 5-hour phase difference as shown (bottom curves), were mixed at the time indicated by the arrow. The rhythm continued, with a phase having its maximum at a time precisely halfway between the maxima of the two original cultures. The middle curve shows the result of mixing cultures having the same phase, done at the time indicated by the arrow. No change of phase was observed. The upper graph shows the result which would be theoretically expected upon mixing two cultures 5 hours out of phase, on the assumption that no interaction was involved. Two "standard" luminescence curves, which were measured from a culture which had not been mixed, were summated with a 5-hour phase difference. For purposes of comparison, the resultant curve is plotted on the graph along with the original standard curve, the latter having been displaced by 2½ hours on the time axis and normalized to the calculated curve. It may be seen that the shape of the calculated curve does not differ greatly from that of the original "standard" luminescence curve.

5 hours), the cultures were mixed in equal proportions, and the luminescence changes in the mixed cultures were measured.

If two typical curves showing the luminescence rhythm are summated, the phase of the two curves being different by five hours (75°), the resultant curve differs only slightly in shape from the original curves (Fig. 10, top). The maximum of the resultant curve lies precisely midway between the maxima of the two original curves.

In the actual mixing experiment, the maximum in luminescence of the mixed cultures occurred halfway between the maxima of the two separate unmixed cultures. Moreover, the shape of the curve from the mixed cultures was very similar to that which was obtained when the measured luminescence of the separate cultures was summated. The mixing experiment therefore indicates that no cellular interaction was involved.

Discussion

The subject of persistent endogenous rhythms has been recently reviewed by Harker (1958), Pittendrigh and Bruce (1957), and Bünning (1956). These reviewers, as well as other authors, have taken the view that the property of rhythmicity may be a nearly universal feature of organisms. This view is derived, largely, from the observation that endogenous rhythms are extremely widespread, having been reported from a large variety of both plants and animals. Furthermore, Pittendrigh and Bruce develop the generalization that most, if not all organisms can measure time; that they possess clocks. They consider that the basic mechanism evolved early, and that it has been retained in the course of evolution as a part of the adaptive organization of all organisms. Their use of the word "clock" refers to the basic mechanism involved in cellular rhythmicity, and the essential properties of this mechanism are considered to be similar in different organisms.

Pittendrigh and Bruce (1957) thus distinguish between the clock as the basic mechanism, and the persistent rhythms which are presumed to be controlled by the clock. Other authors (Brown, Hines, Webb and Fingerman, 1950; Stephens, 1957a; Harker, 1958) have similarly concluded that an overt persistent rhythm may be distinguished from an underlying mechanism, and our studies with *Gonyaulax* give support to this thesis. For example, since it was found that concentrations of compounds taking part in the luminescent reaction could be changed without shifting the phase of the rhythm, it is probable that the luminescence rhythm does not in itself constitute the basic mechanism. Furthermore, we have recently reported a persistent rhythm of cell division in *Gonyaulax* (Sweeney and Hastings, 1957b). The luminescence rhythm and the cell division rhythm have essentially identical properties. Moreover, we have not been able to demonstrate a phase shift in one rhythm which is not accompanied by a similar phase shift in the other rhythm. These findings give additional support to the hypothesis that one basic mechanism controls both rhythms.

The identity and physico-chemical nature of the presumed basic clock mechanism in persistent rhythms remains undefined. But if the properties of this basic mechanism in *Gonyaulax* may be deduced from the rhythm of luminescence, then it is evident that the mechanism possesses essential clock-like properties; the period is not greatly affected by environmental factors, but the phase is labile to resetting by the appropriate external changes. We may note, in addition, that light emission in *Gonyaulax* is clocked so that it is maximal during the night phase, when it is visible; and without environmental inhibition, luminescence is minimal during the day phase. However, since the possible utility of the light emission is not known, the functional significance of clocked luminescence is not apparent.

Many of the characteristics of the rhythm of luminescence which we have described are similar to the characteristics of persistent rhythms in a variety of other organisms, ranging from other unicellular forms to mammals. The comparisons outlined below do not pretend to be complete, but they serve to illustrate the point. The remarkable similarities found support the view of Pittendrigh and Bruce (1957), that the basic mechanism involved in rhythmicity is the same in all organisms.

Practically all the persistent diurnal rhythms described have natural periods which are close to but different from 24 hours. This includes rhythms in *Drosophila* (Pittendrigh, 1954), *Uca* (Webb, Brown and Sandeen, 1954), *Oedogonium* (Bühnemann, 1955a), *Euglena* (Bruce and Pittendrigh, 1956), and many others. The natural period may range, in different organisms, from about 21 to 27 hours. In fact, significant differences in the natural periods in different individual mice are well documented (Pittendrigh and Bruce, 1957).

Studies of rhythms in a variety of organisms, including the bee (Wahl, 1932), *Uca* (Brown and Webb, 1948), *Avena* (Ball and Dyke, 1954), *Drosophila* (Pittendrigh, 1954), and *Euglena* (Bruce and Pittendrigh, 1956), have shown that in each case the period is nearly the same at temperatures which differ by 15° C., or more. It is interesting to note that the effect of temperature upon the period of the *Gonyaulax* rhythm is similar to that reported by Bühnemann (1955b) for the rhythm of sporulation in *Oedogonium,* in that the apparent Q_{10} for both is less than 1.0. Two cases may therefore be interpreted as the result of an over-compensation in the mechanism responsible for temperature independence (Hastings and Sweeney, 1957b).

Only a few experiments have been specifically designed to detect the effect of different light intensities upon the natural period of persistent rhythms. In those cases which have been reported (see Harker, 1958), the natural period has been found to change no more than an hour or two under different light intensities.

The entrainment of rhythms to periods different from 24 hours has been reported in several organisms, including *Euglena* (Bruce and Pittendrigh, 1956) and *Oedogonium* (Bühnemann, 1955a). In these and other cases, as in *Gonyaulax*, the rhythms return to the characteristic natural period when the organisms are returned to constant conditions.

On the other hand, several experiments have been reported in which rhythmic organisms still continue to show a 24-hour rhythm while being subjected to light-dark cycles which differ from 24 hours. For example, Webb (1950) found that the period of the *Uca* rhythm was not changed while the organisms were subjected to light (95 foot-candles) and dark periods of 16 hours each, and Tribukait (1954) found that entrainment to an imposed light-dark cycle occurred in the mouse only so long as the imposed cycles did not differ greatly from the natural period.

Studies with *Gonyaulax* suggest a possible reason for the lack of apparent entrainment in experiments such as those cited above: the light intensities used may not have been sufficiently bright. In *Gonyaulax,* the luminescence rhythm may be entrained to periods which differ greatly from the natural period. Our interpretation of this entrainment is that repetitive phase resetting results in a period corresponding to the imposed schedule. The importance of light intensity as a parameter in phase shifting by single light perturbations has been documented in experiments with *Gonyaulax.* That it is equally important in entrainment has been shown in an experiment with *Gonyaulax* described elsewhere (Hastings and Sweeney, 1958), in which it was found that entrainment occurred at a light intensity of 800 foot-candles, but not at 200 foot-candles.

Entrainment of rhythms to imposed cycles which are only slightly longer or shorter than the natural period has been discussed by Pittendrigh and Bruce (1957). Their interpretation suggests that the mechanism may be different from that involved in entrainment to cycles differing greatly from the natural period.

The role of 24-hour light-dark cycles in establishing the phase of diurnal rhythms has long been recognized, and experiments with many organisms have demonstrated that, as in *Gonyaulax,* the phase shifts in response to a new light-dark cycle which is out of phase with solar night and day. The fact that the light intensity used in such experiments is of importance has been shown by Brown, Fingerman and Hines (1954).

That non-repeated light perturbations are capable of establishing or changing the phase of a persistent rhythm has been stated as an important generalization only in recent years (Pittendrigh and Bruce, 1957), although some previous studies (Kalmus, 1940; Webb, 1950) do provide examples of the phenomenon. The phenomenon provides another analogy between the characteristics of persistent rhythms and the known properties of physical oscillators. It is well known that a single disturbance or perturbation applied to an oscillating system will quite generally shift its phase without any modification to the period, and the behavior of a simple pendulum is a good example. Pittendrigh and Bruce (1957) have found phase shifts following single light perturbations in persistent rhythms of *Euglena* and *Drosophila,* and the rhythm in *Gonyaulax* provides another example of the phenomenon.

Detailed studies on the effect of the duration and intensity of single perturbations have not yet been reported in other organisms, but it appears that the nature of the phase shift in *Gonyaulax* may differ in one respect from that reported for *Drosophila* (Pittendrigh and Bruce, 1957). Following a single light perturbation in *Drosophila* there may occur "transients," so that the phase is not reset immediately but comes to its stable position only after several cycles. In *Gonyaulax,* on the other hand, phase has been found to be reset immediately. The reason for this difference is not known, but it may be related to the relative complexity of the organisms involved.

With respect to the phenomenon of phase shifting, Bruce and Pittendrigh (1957) have discussed whether the resetting signal is the step-up in light intensity (dawn) or the step-down in light intensity (dusk). Several experiments with *Gonyaulax* have adequately illustrated that the phase is labile to both, so that neither event may be said to be the timing cue to the exclusion of the other. For example, the experiments shown in Figure 3 illustrate both a light-to-dark transition followed by constant darkness, and a dark-to-light transition followed by constant light. In both cases, the last transition resulted in a phase shift.

The action spectrum for shifting the phase of the luminescence rhythm by a single light perturbation shows relatively sharp maxima in effectiveness at 475 mμ and 650 mμ (Hastings and Sweeney, unpublished). The red maximum, in particular, suggests that chlorophyll acts as a photosensitizer for phase shifting. Since the effects of single light perturbations are essentially the same in plants and animals, we may conclude that in *Gonyaulax* the photosensitizers involved in phase determination are not a part of the basic mechanism of rhythmicity. In animals, also, the photoreceptor pigments of the eye are not a part of the basic mechanism, although they function in phase determination by light. Whitaker (1940) reported that blinded mice possess a natural period of about 24 hours in their activity rhythm, but that the rhythm could not be entrained by 24-hour light-dark cycles to correspond with solar night and day, as in normal mice.

It is known that temperature changes (Pittendrigh, 1954; Stephens, 1957a), and perhaps certain other factors (Harker, 1958) may also serve to establish or reset phase. There is no report, however, that mechanical disturbances can be effective in other organisms in this regard.

The possibility that individuals in a population may entrain each other was suggested by Pittendrigh and Bruce (1957). However, Stephens (1957b) was unable to demonstrate any significant phase modification in individual fiddler crabs when they were placed together with crabs possessing a different phase. A similar result was found in the present studies with *Gonyaulax*.

It is of interest to note that the shape of the luminescence curve obtained in experiments where *Gonyaulax* cultures possessing different phases were mixed is not greatly different from that for the unmixed cultures. Indeed, as already pointed out, this is the expected result of adding two luminescence curves which are five hours out of phase with one another. Thus, a population composed of cells having at least two different phases is difficult to distinguish from the usual experimental populations, in which we have assumed that all cells possess the same phase. This experiment serves to caution us. In a biological rhythm having a sinusoidal shape, measurements from populations may not accurately represent the behavior of individual cells.

We do not know how the luminescence of the individual *Gonyaulax* cell at different times in the cycle compares with that measured in a population. The question is an important one, and there are several possibilities which, in the absence of any relevant data, need not be discussed here. This problem is being investigated utilizing measurements of the rhythm of cell division, where the performance of an individual cell may be repeatedly and relatively easily scored.

Although several suggestions have been made concerning the physico-chemical nature of the basic mechanism involved in persistent diurnal rhythmicity (Pittendrigh and Bruce, 1957; Hastings and Sweeney, 1958), none has received any substantial support. It is hoped that information concerning the extent and kind of biochemical changes associated with the rhythms will be of value in understanding this basic problem. Studies of this nature are in progress with *Gonyaulax*.

SUMMARY

1. The characteristics of a persistent diurnal rhythm of luminescence in the dinoflagellate *Gonyaulax polyedra* are described.

2. The light emission upon stimulation, from cultures which are kept in alternating light and dark periods of 12 hours each (= LD), is 40 to 60 times greater during the dark period than during the light period. If LD cultures are placed in continuous dim light (100 foot-candles) a diurnal rhythm of luminescence persists. If LD cultures are placed in continuous bright light (> 1500 foot-candles) the rhythm is damped, and no fluctuations occur in the amount of light emitted.

3. The occurrence of rhythmicity is not dependent upon prior exposure to LD conditions. Cultures which have been grown in bright light for as long as one year show a diurnal rhythm when placed in constant dim light or darkness. Cultures kept in alternating light and dark cycles which are greater or less than 24 hours similarly show a diurnal rhythm when returned to constant dim light or darkness. "Training" or "memory" is therefore not involved.

4. The rhythm can be entrained by light-dark cycles which are different from 24 hours. The period of the luminescence rhythm corresponds to light-dark cycles which have periods ranging between 12 and 32 hours.

5. The period of the rhythm is always close to 24 hours when the cells are kept under constant conditions, but it varies slightly depending upon the temperature and light intensity.

6. The phase of the rhythm under constant conditions is related to the time at which the previous light and dark periods occurred. Moreover, the phase may be shifted by interposing a non-repeated exposure to a different light intensity. The number of hours by which the phase is shifted in such an experiment is dependent upon the intensity and duration of the light treatment, and the time in the cycle when it is administered.

7. Exhaustive mechanical stimulation does not alter the phase of the rhythm.

8. When cultures having different phases were mixed, no evidence was found which would indicate that there was any interaction between them.

9. The evidence presented indicates that the diurnal rhythmicity is the consequence of a basic oscillatory mechanism which is inherent to the cell.

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THE ACTION OF INSULIN ON CELLS AND PROTOPLASM[1]

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In a lecture he gave in 1947, Best (1948) stated, "We often hear the statement made that we have had insulin for 25 years and still do not know exactly how it acts. This is quite true, but we know more about the action of insulin than about any other hormone."

Strangely enough, the great science of endocrinology, with its vast body of information concerning the chemistry and the ultimate effects of various hormones, has not been able to solve the basic problem of why the different hormones act as they do. In recent years in the attempt to understand the action of insulin, there has been more and more emphasis on studies of the cell as a whole rather than on studies of fragments or extracts of cells. In a thoughtful review of the literature, Ross (1956) is led to comment (p. 364), "It is apparent that no consistent effect of insulin has been demonstrated in cell-free systems." And the biochemist Levine, a leading authority in the field of carbohydrate metabolism and long a student of insulin, wrote recently (Levine and Goldstein, 1955) (p. 344), "It would be expected therefore that a certain degree of morphological intactness is necessary to demonstrate hormonal effects and actions. Otherwise we would be pulling the trigger of an unassembled gun." Levine himself has turned to the ways of thought and the methods of cell physiology in order to obtain a solution of the insulin problem.

Thus it may not be too presumptuous for cell physiologists to express ideas about the mechanism of insulin action. Indeed back in 1914, Höber suggested that diabetes might be due to a change in the permeability of cells for sugar. This idea was then taken up by Wiechmann (1924, 1926), and by Häusler and Loewi (1925; see also Loewi, 1927). However, the older evidence in favor of the permeability theory was not very convincing, and interpretations in terms of the cell as a whole were pushed into the background by chemical work which seemed to show that insulin had a specific effect on certain isolated enzyme systems. More recently, and on the basis of newer evidence, the permeability theory has been revived and modernized, and it is finding wide support.

No attempt will be made here to review the enormous mass of literature on insulin. Books on endocrinology contain a great deal of information and there have been a number of recent reviews by authorities in the field (Haugaard and Marsh, 1953; Stadie, 1954; Levine and Goldstein, 1955; Weil-Malherbe, 1955; Ross, 1956; Stich and Maske, 1956).

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[1] Supported by a grant from the National Science Foundation.

A few facts stand out. Some of these have been long known and were mentioned in a review published by Macleod in 1924. For our purposes, it may be well to remind the reader that:

1. The action of insulin in reducing the concentration of sugar in the blood is not due to an action on the blood itself, for insulin does not have this effect on blood withdrawn from the body. Hence the cells and the protoplasm they contain must play a part in the lowering of sugar concentration in the blood.
2. The convulsive action caused by excess insulin is not due to an effect on the cerebrum, for it occurs in decerebrate animals.
3. Lowering of the sugar concentration in the medium surrounding isolated nerve or muscle has no effect in stimulating either nerve or muscle.
4. When a rabbit is given a lethal dose of insulin, violent convulsions occur; these are then followed by a comatose stage, and convulsions and coma continue in alternate phases until, after an hour or more, the animal dies.
5. The harmful results of excess insulin can be relieved by the injection into the blood stream of a small amount of glucose.
6. Lack of insulin causes a failure of fat, carbohydrate and protein formation. Best (1953) sums up the situation (p. 434) by stating that "insulin is a central anabolic hormone without which many of the building processes . . . cannot proceed at the physiologic rate."
7. Insulin also causes an increase in the rate of oxidation of sugar. Thus it is a catabolic hormone as well as an anabolic hormone.

It is clear that insulin markedly increases the rate of activity of various enzymic actions, and yet when purified preparations of these enzymes have been tested, there seems to be little or no effect of insulin upon them. Moreover it would be hard to explain how it would be possible for a single substance to have a direct effect on all the various enzymes responsible for the synthesis of carbohydrates, proteins and fats, as well as those responsible for the oxidation of sugar. Hence we apparently must conclude that in one way or another some change in the cell or its protoplasm has an accelerating effect on many types of enzyme activity.

At the present time, what is doubtless the leading theory of insulin action holds that the primary effect of insulin is to change the cell in such a way as to facilitate the passage into it of various sugars. This theory, due in its present form to Levine and his collaborators, has been supported not only by the work of Levine and his group, but also by the careful and ingenious experiments of various other investigators. Perhaps the most impressive work is that of Park, Bornstein and Post (1955). The entire subject is ably reviewed by Ross (1956), and this review should be consulted by anyone interested in details or references to the rather extensive literature. The permeability theory has been enthusiastically endorsed by Stadie (1957), who is certainly one of the outstanding investigators in the field.

Scarcely anyone has attempted to criticize the permeability theory, although such criticism is possible, both from the standpoint of our knowledge of permeability and transport mechanisms, and also because the theory can scarcely offer an explanation of some of the basic known facts of insulin action. This latter point will be discussed in a later section.

Effect of Insulin on the Permeability to Glucose

Our experiments with insulin were done entirely on relatively simple, isolated cells of lower organisms. These cells offer exceptionally favorable material for the cell physiologist, but work with such cells is open to the criticism that the action of insulin may be confined to the cells of vertebrate animals. Such an opinion is held by Ross in the review cited above, and it was expressed forcibly by Best, Jephcott and Scott (1932). However, there are reports of insulin action on the cells or tissues of protozoa, sponges, flatworms, crabs and insects; on yeast cells, and on various kinds of bacteria. Whether this literature is sound or not is a question we do not care to discuss. Certainly there can be no objection to our using simple living cells to explain insulin action, for if we can obtain effects on these cells with insulin and if such effects can be used to interpret the action of insulin in higher animals, we may be able to offer suggestions of some value.

For the study of cells and protoplasm the eggs of marine invertebrates offer many advantages and this type of material has often been used by cell physiologists. We used the eggs of the sea urchin *Arbacia punctulata,* the surf clam *Spisula solidissima,* and the annelid worm *Chaetopterus pergamentaceus.* All of these eggs are readily available at Woods Hole. If insulin directly favors the entrance of glucose into cells (quite apart from any indirect effect it might have as a result of the utilization or combination of glucose within the cell), we thought that perhaps we might be able to find evidence for such a direct effect on marine egg material. This we were unable to do, and our results were wholly negative. For this reason we shall not attempt to report them in any detail, but will merely cite a few of our experiments as briefly as possible.

One of the standard ways of determining the ease with which dissolved substances pass through cell membranes is to study the osmotic behavior of cells in relation to solutions of the substances in question. There are a variety of such osmotic methods. One of the simplest of these methods depends on the fact that the more readily a substance penetrates, the less osmotic pressure it can exert against the plasma membrane of the cell. By observing the changes in volume of the cells when they are immersed in various concentrations of a given substance, one can obtain a rapid measure of the ease with which a substance enters. In our particular case, if insulin favored the entrance of glucose, a solution of glucose containing insulin would be less potent osmotically than a similar control solution which differed only in lacking insulin. In order to obtain as great an effect as possible, we used saturated solutions of insulin. These were obtained by dissolving 0.2 mg. of insulin in one ml. of the glucose solution. Actually not all of the insulin went into solution. All the solutions were brought to the pH of sea water. The insulin we used was a preparation which was relatively zinc-free; it was kindly supplied by the Eli Lilly Company through the kindness and courtesy of Dr. G. H. A. Clowes. In view of the fact that many types of protoplasm are very sensitive to zinc, we were indeed fortunate to obtain this preparation from which 98.8% of the zinc ordinarily present in crystalline insulin had been removed. Actually an assay made by the Eli Lilly Company showed only 0.0061% zinc in the dry material.

Table I shows the results of our experiments on sea urchin eggs. In this table, as also in Tables II and III, the ± sign indicates standard deviation. A molar solution of glucose caused a slight decrease in the volume of the eggs. In the

TABLE I

Effect of glucose and glucose + insulin on the osmotic behavior of Arbacia eggs. Measurements of diameters were made after the eggs had been immersed in the solutions for 5 minutes. The values show the average of 10 measurements

Diameter of eggs in sea water	77.8 ± 2.02 microns
Diameter of eggs in molar glucose solution	71.5 ± 2.18 microns
Diameter of eggs in molar glucose solution containing insulin	70.7 ± 2.29 microns

presence of insulin approximately the same decrease occurred. If insulin had favored the entrance of the glucose, then the solution of glucose containing the insulin should not have caused as great a shrinkage.

Similar results were obtained with eggs of the clam *Spisula*, as is shown in Table II. Again there is no indication that insulin favors the entrance of glucose.

In our experiments with eggs of the worm *Chaetopterus*, we ran into difficulty. When we immersed these eggs in solutions of glucose, the sugar entered rapidly, so rapidly in fact that even solutions are strong as 2 *M* caused no shrinkage of the eggs. We experimented with 2 *M* and 1.75 *M* and 1.5 *M* glucose solutions with and without insulin. In all cases, in the absence of insulin the glucose entered more rapidly than when it was present. In the 1.75 *M* and 1.5 *M* glucose, frequently the eggs swelled so rapidly that they broke. This sometimes made measurements uncertain. Apparently in the glucose solutions, absence of ions like calcium changed the semipermeable membrane of the cell in such a way that it became permeable to glucose. Perhaps the small amount of zinc in the insulin helped partially to stabilize the membrane. Because of the increased permeability of the cell membrane in the absence of the salts of sea water, we decided to compare the behavior of glucose and glucose + insulin in the presence of an appreciable amount of sea water. The results are shown in Table III. In this case also, presence of insulin does not favor the entrance of glucose.

Our results with marine eggs lead to the conclusion that the effect of insulin in increasing the rate of entrance of glucose into cells is not a general phenomenon true for all types of living material. Other authors in the past have reached the same conclusion. Thus it is now commonly held that insulin does not increase the rate of entrance of glucose into the erythrocytes of man and mammals. However, the literature on erythrocytes is, or at least has been, highly controversial (see Foshay, 1925; Häusler and Loewi, 1925; Loewi, 1927; Högler, Thomann and Überrack, 1929; Himmerich and Tschernjak, 1936; also many papers cited by them; Wilbrandt, 1947; Guensberg, 1947; Pletscher, von Planta and Hunzinger.

TABLE II

Effect of solutions of glucose and glucose + insulin on the osmotic behavior of Spisula eggs. Measurements of diameters were made after the eggs had been immersed in the solutions for 5 minutes. The values are in microns; they show the average of 10 measurements

	0.9 *M*	0.8 *M*	0.7 *M*
Diameter of eggs in sea water	61.0 ± 2.28	56.8 ± 1.67	56.4 ± 1.07
Diameter of eggs in glucose	56.8 ± 1.78	56.4 ± 1.52	58.9 ± 0.83
Diameter of eggs in glucose + insulin	53.7 ± 1.77	53.5 ± 3.80	54.1 ± 2.87

TABLE III

Effect of glucose and glucose + insulin on the osmotic behavior of Chaetopterus eggs.
Solution A = 4 parts molar glucose solution + 1 part sea water.
Solution B = 4 parts molar glucose solution containing insulin + 1 part sea water

Control eggs in sea water measure 99.4 ± 2.06 microns

Eggs in A (glucose alone) measure		Eggs in B (glucose + insulin) measure	
After 3 minutes	94.0 ± 1.63 microns	After 3 minutes	94.2 ± 1.83 microns
After 136 minutes	95.4 ± 2.50 microns	After 135 minutes	94.0 ± 2.58 microns

1955). Any interpretation of this literature is complicated by the fact that glycolysis and other changes in carbohydrates may well occur in blood cells. Park and Johnson (1955) failed to find any increase in the rate of entrance of glucose and galactose into rat brain cells when insulin was present, but here, too, the results are based on the assumption that under the conditions of the experiment both glucose and galactose remained unaltered when they entered the cells, and this conclusion may not be entirely warranted (compare Sols and Crane, 1954).

If, as is now commonly believed, the favorable effect of insulin on the transport of sugar into cells is due to some sort of an enzyme-controlled reaction, then acceleration of this transport promoting enzyme might well be the cause of the increase in the rate of entrance of sugar. Thus the more rapid transport of sugar into cells in the presence of insulin might merely represent one aspect of the general effect of insulin in accelerating diverse types of enzyme activity. In other words, the more rapid entrance of sugar, instead of being the basic reason for insulin action, might be a result rather than a cause of some underlying change that is responsible for a general increase in enzymic activity. What could such a cause be?

EFFECT OF INSULIN ON COLLOIDAL CHANGES IN PROTOPLASM

When various types of cells are excited by stimuli of one sort or another, calcium is released from the cell cortex, and this calcium then activates a proteolytic enzyme system. The proteolytic enzyme also serves as a clotting enzyme and produces a gelation of protoplasm in the interior of the cell. This gelation involves an oxidation of –SH to S–S groups. Thus the release of calcium can result in an increase in protease activity and also an increase in cellular oxidations. In other words, calcium release is the trigger that starts off a number of enzymic reactions. The evidence on which these statements is based has been presented in considerable detail in recent books (Heilbrunn, 1956, 1958); see also Wilson and Heilbrunn, 1957.

Could it be possible that in one way or another insulin might act in somewhat the same way that stimulating agents do, and what could conceivably be the reason for such an action? This possibility is what intrigued us and induced us to undertake the work that is described below.

If gelation and the reactions underlying gelation in protoplasm constitute the trigger for protoplasmic activity, then presumably protoplasm must have some method of braking or inhibiting the gelation. In the books just referred to, strong evidence is presented to show that heparin and heparin-like substances can constitute such a brake. Heparin not only can inhibit protoplasmic gelation in much the same way that it inhibits blood clotting, it can also inhibit the action of various types of enzymes. In living cells generally, there seems to be a balance between the factors which tend to induce gelation or clotting and those which tend to prevent it. Heparin (and/or heparin-like substances) is one of the inhibiting factors. If we could imagine a substance which would antagonize or neutralize the effect of heparin and similar substances, then it might well act to accelerate various enzyme systems in the protoplasm. Insulin is such a substance, as we shall now attempt to show.

Sol-gel reactions undoubtedly occur in many, if not all types of protoplasm, but they are especially evident in the ameba. Moreover, in the ameba, a small amount of heparin can be shown to prevent the clotting reaction which normally occurs whenever the cell is torn or broken, that is to say the surface precipitation reaction. We use the giant ameba, *Chaos chaos,* and the heparin we used in our experiments was kindly supplied by the Upjohn Company. If an ameba is immersed in a dilute solution of heparin, say a 0.01% solution, and the ameba is crushed by exerting pressure on the coverslip over the animal, no surface precipitation reaction occurs and the contents of the ameba flow out through the solution. However, if the heparin solution is also made to contain a 0.01% solution of insulin, there is an excellent surface precipitation reaction and the exuding protoplasm forms a distinct membrane about itself.

We performed a series of experiments in which various concentrations of insulin were balanced against various concentrations of heparin. In deciding whether or not a surface precipitation reaction occurs, it is important not to vary too greatly the amount of pressure with which the ameba is broken. For with too great pressure and with too rapid emergence of the interior protoplasm, there is scarcely time for a proper reaction to occur. It is difficult to measure the amount of pressure applied to a coverslip. In order to measure this pressure, one of us (Ashton) devised an apparatus in which a small rectangular piece of glass was attached to a lever which in turn was attached to a DeNouy tensiometer. With this apparatus it is possible to measure the amount of pressure applied before a cell breaks. The measurements are not very exact, but they have the advantage of being objective. For cells which do not vary greatly in volume, as for example sea urchin eggs, the pressure required to break them, as indicated by our apparatus, is reasonably constant. However, for amebae which differ markedly in size, as do our specimens of *Chaos chaos,* the breaking pressure varies more widely, for with the larger amebae there is more resistance to the pressure imposed upon them. Table IV shows what happens when amebae are broken in mixtures of insulin and heparin. The last column indicates whether or not a surface precipitation reaction occurred and whether it was a strong or a weak reaction. The amount of pressure required to break the amebae is also recorded. As was to be expected, this pressure varied widely, but whether the pressure was relatively great or relatively small, the results were always the same. It is clear, therefore, that the effect of heparin in preventing the protoplasmic clotting necessary for the surface precipitation reaction can be completely blocked by the addition of insulin. Moreover, a control test showed that the amount of zinc present in our solutions had no such effect. It should be noted that a given amount of insulin can neutralize four times as much heparin.

Further evidence of a combination between insulin and heparin is provided by experiments in which it was shown that the metachromatic reaction of heparin with toluidine blue was prevented by solutions of insulin. In these experiments, shown in Table V, relatively large amounts of insulin were necessary to block completely the metachromatic reaction. Here we are dealing with a system in which only insulin and heparin are present, whereas in the earlier experiments the system included not only insulin and heparin, but also the protoplasm of the ameba. Probably the protoplasm, or rather some proteins contained in it, have an affinity for heparin and can unite with it in spite of the presence of insulin. At any rate, this might constitute an explanation of the different types of ratios obtained in the two experiments. Another explanation might be that it may take more insulin to block

TABLE IV

The effect of mixtures of heparin and insulin on the surface precipitation reaction of Chaos chaos

% insulin	% heparin	Ratio insulin/heparin	Pressure in milligrams	Spr
0.01	0.01	1–1	120	strong
		1–1	88	strong
		1–1	92	strong
		1–1	120	strong
		1–1	160	strong
0.01	0.02	1–2	208?	strong
		1–2	140	strong
		1–2	116	strong
		1–2	52	strong
		1–2	96	strong
0.01	0.03	1–3	116	weak
		1–3	124	strong
		1–3	188	weak
		1–3	48	weak
		1–3	116	weak
0.01	0.04	1–4	48	weak
		1–4	168	weak
		1–4	128	weak
		1–4	252?	very weak
		1–4	76	very weak
0.01	0.05	1–5	96	none
		1–5	60	none
		1–5	134	none
		1–5	68	none
2×10^{-7} *M* zinc	0.01		124	none
			125	none
			144	none
			172	none
			145	none

TABLE V

Metachromatic reaction of mixtures of heparin and insulin. One milliliter of a 0.02% insulin solution was mixed with an equal volume of various concentrations of heparin, and the various mixtures were then tested for metachromasia with 6 drops of a 0.01% solution of toluidine blue

% insulin	% heparin	Ratio insulin/heparin	Reaction
0.02	0.02	1	+
0.02	0.01	2	+
0.02	0.0067	3	+
0.02	0.005	4	+
0.02	0.004	5	+
0.02	0.0033	6	+
0.02	0.00286	7	+
0.02	0.0025	8	+
0.02	0.0022	9	+
0.02	0.002	10	+
0.02	0.00182	11	+?
0.02	0.00167	12	+?
0.02	0.00154	13	–
0.02	0.00142	14	–
0.02	0.00134	15	–

the metachromatic reaction of heparin than it does to block its effect on clotting or on enzymic action.

When amebae are stained with toluidine blue, the outer region of the cell gives a beautiful metachromatic color, a color such as that which would be given by heparin or a heparin-like substance. But if amebae are immersed in solutions of insulin for some hours, staining with toluidine blue no longer gives a metachromatic reaction. Such a loss of the metachromatic reaction occurs even in very dilute solutions of insulin. This is shown in Table VI. In interpreting this table, it should be remembered that the amebae were immersed in solutions whose volume was very large in comparison with the volume of the amebae. Actually in the experiments reported in the table, 10 ml. of solution were used and only a few drops of a concentrated suspension of amebae.

Our experiments indicate that insulin can and does combine with heparin. There is some indication in the chemical literature in support of this view. According to Gorter (1954), heparin can combine with various proteins, including insulin. In Gorter's experiment, the insulin was combined with the lipid cephalin

TABLE VI

Metachromatic reaction of Chaos chaos after the amebae were immersed for 16 hours in various concentrations of insulin solution

Concentration of insulin, %	Reaction
0	+
0.000625	+
0.00125	–
0.0025	–
0.005	–

(phosphatidyl-serine), and Gorter believes that as a result of the complex formed between insulin and heparin, the lipid is set free. The reaction between heparin and insulin is strongly influenced by hydrogen ion concentration, a fact which may be of considerable importance in the interpretation of biological phenomena.

DISCUSSION

Although the permeability theory of insulin action has been so widely accepted, as already noted, the fact that insulin increases the rate of passage of sugar into a cell could well be the result of some acceleration of an enzyme responsible for such transport, so that the more rapid entrance of the sugar would really be a result rather than a cause of enzyme action. A somewhat similar idea was expressed many years ago by Staub (1927). But this type of objection is perhaps not too serious. The value of a theory lies in the extent to which it can explain and interpret known facts. Perhaps the most important fact about the action of insulin is that it behaves as an anabolic hormone and produces an increase in the synthesis of carbohydrates, proteins and fats. It could of course be claimed that inasmuch as insulin hastens the entrance of sugar into a cell, this fact in itself might favor the synthesis of proteins, for the energy for such syntheses is now believed to come from the oxidation of carbohydrate. This may well be the correct explanation. However, according to Sinex, MacMullen and Hastings (1952), the addition of glucose tends to prevent the insulin-induced synthesis of protein in the rat diaphragm. And if we consider the long-known facts concerning the effects of insulin, the permeability theory would have some difficulty in offering a complete explanation. For if the primary effect of the hormone is to increase the sugar content of a cell, then logically one should be able to imitate the effects of insulin merely by feeding or injecting excess glucose, for such glucose would also increase the cellular sugar. But administration of excess sugar is hardly a cure for diabetes. Moreover, under conditions in which there is an excess of insulin in the blood, if the sugar permeability theory were correct, from a logical standpoint the very worst possible treatment to offset the harmful effects of the excess would be to feed or inject glucose; for the permeability theory assumes that insulin acts by introducing more glucose into the cells. And yet, as is standard knowledge, the convulsions and other adverse symptoms induced by excess insulin can be cured readily enough by the administration of glucose.

Of course in an animal as complicated as a mammal, there are too many interactions of various organ systems to enable one to reach unassailable conclusions by logic based on the behavior of any one organ system. It is quite possible that insulin and sugar have one effect on muscle and another on the cells of the medulla and spinal cord. Thus it might be postulated that in insulin shock, the nerve cells in the basal part of the brain come to lack glucose and that this lack is responsible for the convulsions and the coma. Then the administration of sugar might quickly restore the nerve cells to their normal state.

On the basis of our theory, if insulin acts primarily by combining with heparin and counteracting its effects, then such an action would immediately accelerate various types of enzymic activity. For, as is well known, heparin is an inhibitor of some proteases. Thus, it inhibits the action of trypsin (Horwitt, 1940), and pepsin (Marini and Levey, 1955). It also inhibits the action of ribonuclease

(Zöllner and Fellig, 1953), and of amylase (Myrbäck and Persson, 1952). Because it is a polyanion, it can have a retarding effect on the activity of various enzymes (Spensley and Rogers, 1954). When injected into the blood stream, it tends to extract lipases from tissues (Iselin and Schuler, 1957), and presumably this would retard cellular lipase activity. And inasmuch as it retards the activity of ribonuclease, it might also tend to prevent the synthesis of ribonucleoproteins; this might be a factor in retarding the formation of proteins with enzymic activity. Finally, because heparin prevents the clotting of protoplasm, and such clotting, as stated previously, acts as a trigger for oxidative reactions, it might also retard the oxidative activity of a cell. Hence by the simple combination with heparin, insulin could exert many of the effects we know it to have. In support of our point of view, it might be noted that according to Bond and Spitzer (1955), much of the hypoglycemic effect of insulin is lost if it is injected into rabbits previously injected with heparin. Bond and Spitzer do not believe that this phenomenon is due to any combination of heparin with insulin, for when they injected a mixture of the two substances, insulin action remained unimpaired. But within blood, and even more within cells, various factors such as pH, ionic strength or even the presence of protein co-factors, might have an influence on any possible combination.

As recent authors are coming to realize (Weissbecker and Hitzelberger, 1953; Riley, Shepherd, West and Stroud, 1955), heparin has many physiological actions in addition to its effect on blood clotting. Thus Riley *et al.* suggest "that the function of heparin may be concerned rather with events in the tissues than with the coagulability of the circulating blood," and a similar statement is also made by Weissbecker and Hitzelberger. One interesting phenomenon is the fact that heparin antagonizes the effects of ACTH and cortisone. As we learn more about the heparin and heparin-like substances that are found in cells, we may gain additional insight into life processes and the action of various drugs on these processes.

Obviously the work we have done represents only a beginning. If the theory we propose is correct, then much more work needs to be done in order to place it on a firm footing. Any theory which attempts to give a complete explanation of insulin activity is faced with many difficulties.

SUMMARY

1. Insulin does not speed the entrance of glucose into the eggs of a sea urchin, a clam and a worm.

2. Dilute solutions of heparin prevent protoplasmic clotting in ameba. This action of heparin is blocked by insulin.

3. Evidence is presented to show that insulin combines with heparin. It blocks the metachromatic reaction that heparin gives with toluidine blue. This can clearly be shown *in vitro,* and it is also indicated by studies on living amebae.

4. Earlier work has shown that heparin acts as an inhibitor of various enzymes, and in general it may be thought of as constituting a brake on many of the chemical activities of a cell. By preventing this inhibiting action, insulin is able to promote the synthesis of various essential constituents of the protoplasm.

5. Also, in view of the fact that protoplasmic clotting involves oxidation and can act as a trigger for oxidative activity, insulin by preventing the anticlotting action of heparin can promote oxidations.

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THE EFFECTS OF CERTAIN NEUROHUMORS AND OF OTHER DRUGS ON THE VENTRICLE AND RADULA PROTRACTOR OF BUSYCON CANALICULATUM AND ON THE VENTRICLE OF STROMBUS GIGAS[1,2]

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Krijgsman and Divaris (1955) called attention to the need for pharmacological information about the heart of *Busycon canaliculatum*. Such information was gained in the course of investigations of the physiology of *Busycon*, carried out between 1953 and 1956, and is presented here. For the sake of comparison, experiments on the ventricle of *Busycon* were repeated on the ventricle of *Strombus gigas*. Further experiments with the *Strombus* heart are also reported here. The *Busycon* radula protractor (recommended for physiological investigation by Herrick, 1906) was used for a comparison of the effects of the same drugs on non-cardiac muscle.

I wish to thank Professor John H. Welsh for the suggestion which led to this study and for his guidance.

Pharmacology of the ventricle

METHODS

The amplitude of heart beat was measured on kymograph records from isolated ventricles, perfused with sea water through the auricle in a manner similar to that described by Welsh and Smith (1949) for larger crustacean hearts. The bath was so arranged that it could be flushed with sea water while the ventricle was washed through the cannula between tests. Drugs in sea water solution were applied by substitution for the perfusion fluid. Experiments on the *Busycon* ventricle were carried out at room temperature of 23° C., and experiments on the *Strombus* ventricle were carried out at room temperature which varied between 20° and 25° C.

RESULTS

Acetylcholine produced a decrease in amplitude of beat in the *Busycon* ventricle at a 10^{-9} molar concentration, with diastolic arrest at 10^{-7} molar (Fig. 1, A). The

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[1] This investigation was supported in part by a predoctoral fellowship, HF-5211, from the National Heart Institute, Public Health Service.

[2] This paper, in slightly different form, comprised part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Harvard University.

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[4] Contribution Number 249 from the Bermuda Biological Station. Assisted by a National Science Foundation grant-in-aid through the Bermuda Biological Station.

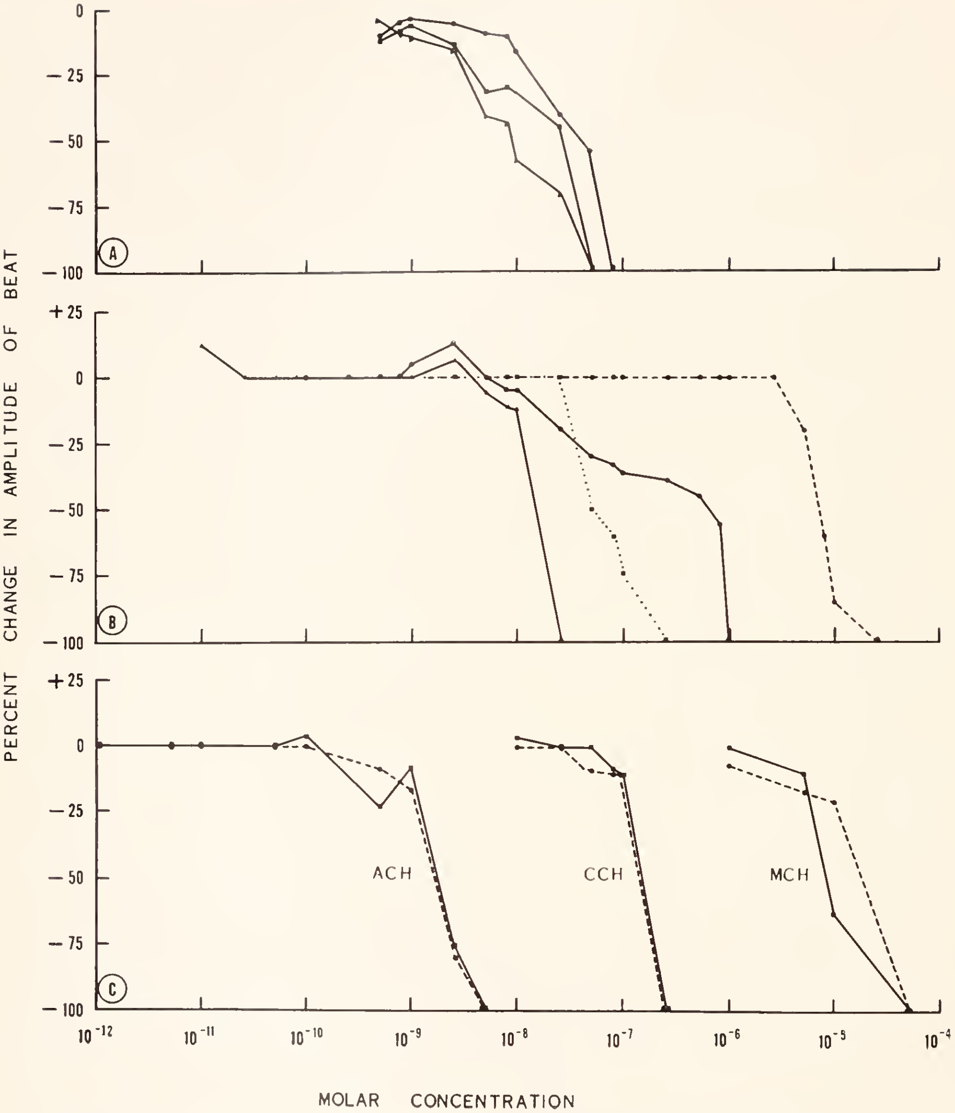

FIGURE 1. A. The effect of acetylcholine on three *Busycon canaliculatum* ventricles. Each point represents the average of the responses of a ventricle to two exposures to the same concentration, once in an ascending series, and once in a descending series. B. The effect of acetylcholine (solid lines), carbamylcholine (dotted line), and acetyl-beta-methylcholine (broken line), on the *Strombus gigas* ventricle. C. The effect of eserine on the concentration-action curve of the *Strombus gigas* ventricle for acetylcholine (ACH), carbamylcholine (CCH), and acetyl-beta-methylcholine (MCH). In each case the solid line represents the effect of the ACH or ACH analogue in the perfusion sea water of an uneserinized heart. The broken line represents the effect of the ACH or ACH analogue applied in perfusion fluid, consisting of a 10^{-5} molar sea water solution of eserine, to a ventricle previously soaked for an hour in 10^{-5} molar eserine.

threshold to acetylcholine was found to be approximately the same for *Strombus,* but the response to a concentration just above threshold was an increase in amplitude, with a decrease in amplitude elicited by concentration ten times threshold (Fig. 1, B). In both *Strombus* and *Busycon,* acetylcholine improved irregular beating at concentrations ten times less than the level of the threshold for an effect on amplitude.

Although carbamylcholine in low concentrations failed to produce the increase in amplitude of the *Strombus* ventricle beat that was seen with acetylcholine, it produced decrease in amplitude in the neighborhood of 5×10^{-8} molar concentration (Fig. 1, B).

Acetyl-beta-methylcholine has an acetylcholine-like effect on the *Strombus* ventricle but with a threshold concentration one thousand times greater (Fig. 1, B).

Eserine failed to potentiate the action of acetylcholine, carbamylcholine, or acetyl-beta-methylcholine on the *Strombus* ventricle. It did regularly abolish the excitatory effect of low concentrations of acetylcholine (Fig. 1, C).

Both adrenalin and noradrenalin proved to have a positive tonotropic effect on the *Busycon* ventricle but the effective concentrations were not in the extremely dilute range at which acetylcholine became effective. At a 10^{-5} molar concentration either neurohumor increased the amplitude of beat about fifty per cent, but the amplitude was increased nearly one hundred per cent when a 10^{-5} molar concentration was obtained as the sum of the molarities of adrenalin and noradrenalin added simultaneously (Fig. 2, A).

5-Hydroxytryptamine was found to have an action on the *Busycon* heart similar to that of adrenalin but with a threshold in the vicinity of 10^{-9} molar, and is thus active in dilutions comparable to acetylcholine dilutions. The *Busycon* ventricle is a thousand times less sensitive to tryptamine than to 5-hydroxytryptamine (Fig. 2, C). Adrenalin or noradrenalin concentrations fifty times greater than threshold concentration for a particular heart irreversibly stop the heart, but 5-hydroxytryptamine at 10^{-2} molar, ten million times the threshold concentration, does not even produce systolic arrest.

In contrast to the synergistic effect of simultaneous addition of adrenalin and noradrenalin to the perfusion fluid, when adrenalin and tryptamine are added to the perfusion fluid simultaneously the effect is not significantly greater than if the same molar concentration were made up of one drug (Fig. 2, B).

5-Hydroxytryptamine acts on the *Strombus* ventricle over a wide range of concentrations with a threshold at 10^{-10} molar (Fig. 3, A).

The antagonism between the negative tonotropic effect of acetylcholine and its analogs, and the positive tonotropic effect of 5-hydroxytryptamine, on the *Strombus* ventricle, is plotted in Figure 3, B, in terms of the reduction by acetylcholine, carbamylcholine, or acetyl-beta-methylcholine of the amplitude maintained by 10^{-7} molar 5-hydroxytryptamine. Acetylcholine acts on the 5-hydroxytryptamine excited ventricle much as on the normal ventricle, but carbamylcholine, which is less effective than acetylcholine in depressing the spontaneous heart beat, is almost as effective an antagonist of 5-hydroxytryptamine as is acetylcholine. Gramine is another antagonist of the action of 5-hydroxptryptamine on the *Strombus* ventricle, and will completely block the action of 10^{-7} molar 5-hydroxytryptamine at a 5×10^{-5} molar gramine concentration.

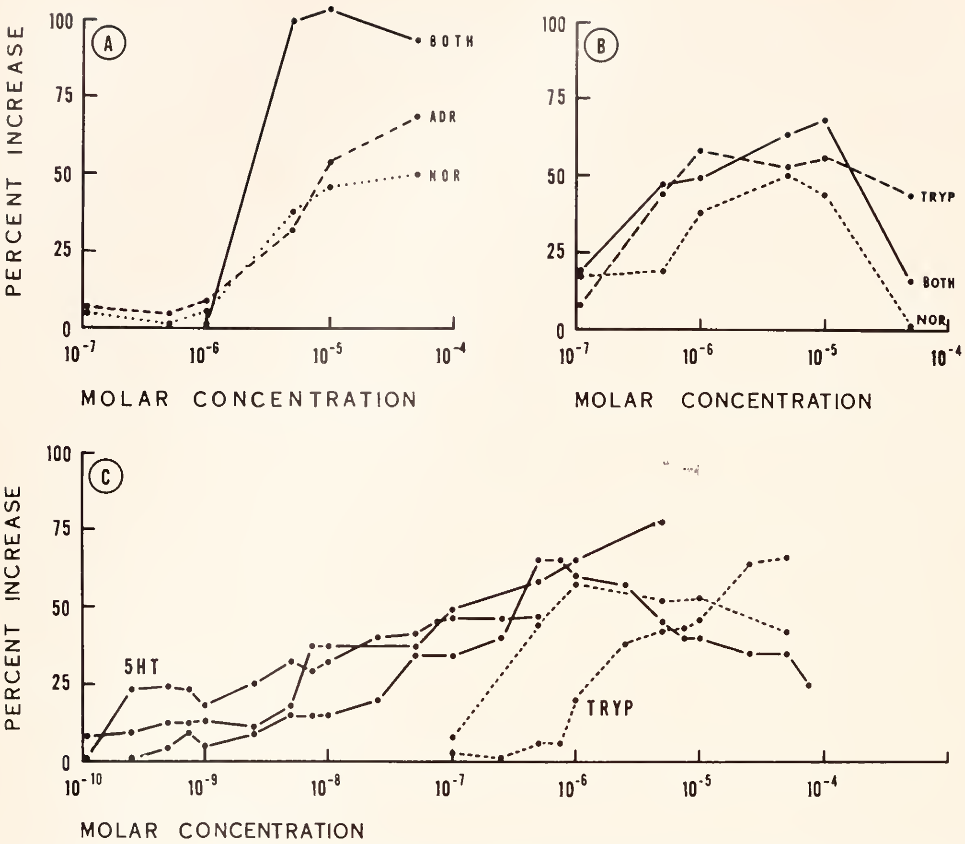

FIGURE 2. A. The effects of adrenalin, of noradrenalin, and of the same molar concentrations made up with equal amounts of the two amines, on the amplitude of beat of the isolated *Busycon canaliculatum* ventricle. Each curve ends at the concentration at which the ventricle stopped in systole. B. A similar comparison of the effects of tryptamine, of noradrenalin, and of both simultaneously on the *Busycon canaliculatum* ventricle. C. The effects of tryptamine and 5-hydroxytryptamine on the amplitude of beat of the isolated *Busycon canaliculatum* ventricle.

DISCUSSION

The pharmacological relations of the *Busycon canaliculatum* ventricle resemble those of other gastropod hearts. Acetylcholine has been shown to depress the beat of the hearts of the gastropods *Buccinum undatum* and *Cyprina islandica* (Welsh, 1956), *Dolabella auricula* (Ebara, 1955), *Cochlitoma zebra* (Divaris and Krijgsman, 1954), *Helix pomatia* (Jullien and Ripplinger, 1950), and *Murex trunculus* (Jullien and Morin, 1931).

Among the hearts listed above, *Murex trunculus* has been reported by Morin and Jullien (1930) to have a small group of nerve cell bodies near the location where Carlson (1905) reports a ganglion in *Busycon*. However, Divaris and Krijgsman (1954) not only found no nervous elements in the white spot at the *Cochlitoma zebra* ventriculo-aortic junction, but also demonstrated the existence of

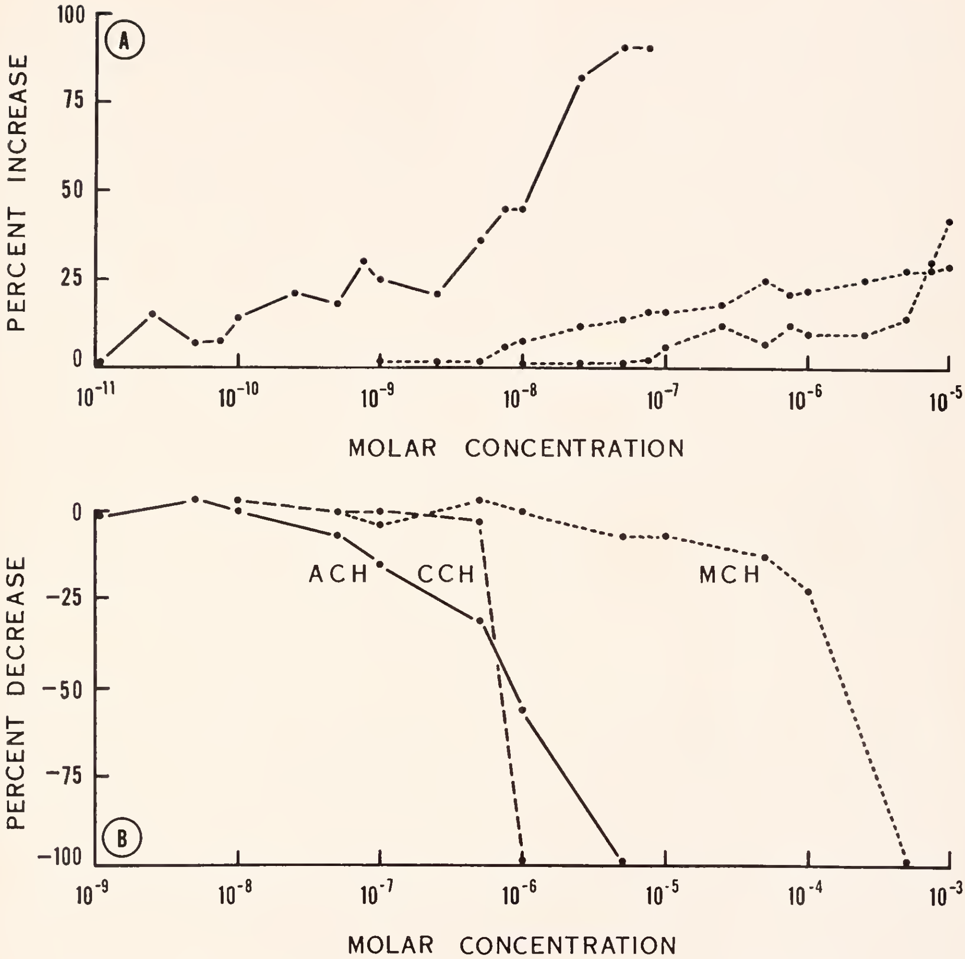

FIGURE 3. A. The effect of 5-hydroxytryptamine on the amplitude of beat of the isolated ventricles of *Strombus gigas* (solid line) and *Aplysia protea* (broken line). B. The effects of acetylcholine, carbamylcholine, and acetylbetamethyl choline, on the amplitude of beat, of the *Strombus gigas* ventricle, which is maintained by perfusion with 10^{-7} molar 5-hydroxytryptamine.

myogenic pacemakers. A myogenic origin for the beat of the heart of *Murex trunculus* was indicated when Cardot, Jullien and Morin (1929) showed that isolated fragments would beat in sea water. Thus, the *Busycon* ventricle reacts to acetylcholine like the hearts of other gastropods which have been demonstrated to have myogenic pacemakers.

The extremely low concentrations at which acetylcholine and 5-hydroxytryptamine are effective on the *Busycon* ventricle are in accordance with Welsh's (1957) finding that they act as neurohumors in *Venus mercenaria*. That 5-hydroxytryptamine is effective in lower concentration than adrenalin or noradrenalin, with an equally rapid onset of action, suggests that 5-hydroxytryptamine might be closer in structure to the natural cardio-regulatory neurohumor of *Busycon* than are the mammalian neurohumors. 5-Hydroxytryptamine has, in fact, been found in the pooled ganglia of *Busycon* by Welsh (1954).

The response of the *Busycon canaliculatum* ventricle to adrenalin and noradrenalin is evidence that it is more similar pharmacologically to the hearts of the molluscs *Eledone cirrosa* and *Anodonta* (Fänge and Ostlund, 1954), which respond by an increase in amplitude of beat, than to that of *Aplysia dactylomela,* which is insensitive to the two neurohumors, as reported by von Euler, Chavez and Teodosio (1952). That the isolated *Busycon* heart beats so well is also a contrast to the *Aplysia dactylomela* heart which will beat spontaneously only if adjacent ganglia are isolated with it (von Euler, Chavez, and Teodosio, 1952). However, I have found the isolated ventricle of *Aplysia* to beat spontaneously, although not well. Its beat may be sustained by ergonovine or by 5-hydroxytryptamine, but its threshold to 5-hydroxtryptamine is between 10^{-8} and 10^{-7}, which is considerably higher than the thresholds of the *Busycon* and *Strombus* ventricles (Fig. 3, A).

Pharmacology of the leached ventricle

Following Burn's theory (1950) of the relation of local hormones to cardiac automatism, it might be expected that a denervated ventricle, which had been deprived, by leaching, of previously synthesized neurohumors, would respond by contraction to either acetylcholine or 5-hydroxytryptamine.

METHODS

In order to ascertain the upper limit to the time an isolated *Busycon canaliculatum* ventricle may remain viable and useful for bioassay, six isolated entire hearts were set aside in sea water at 9° C. for periods ranging from one to six weeks. At intervals, a ventricle was removed to room temperature, allowed five hours for adjustment, and then perfused. It would seem probable that after a week the cut distal portions of the cardioregulatory nerves (from the visceral ganglion; Carlson, 1905) would have degenerated, so that the leached ventricle might react to pharmacological agents primarily as a muscle preparation.

RESULTS

Of two hearts kept at 9° C. for one week, both survived and both beat normally when perfused. That is, after one-half hour one was beating at 18 systoles per minute, the other at 21 and both were emptying completely at each systole. Each continued at its original rate for 5 hours, at the end of which time one showed a threshold response to 5×10^{-9} *M* 5-hydroxytryptamine and 10^{-9} *M* acetylcholine, and the other a threshold response to 10^{-9} *M* acetylcholine and to 10^{-9} *M* 5-hydroxytryptamine.

Of the two hearts set aside for three weeks, only one survived. The other, after one-half hour of perfusion, was beating regularly at a beat of 22 systoles per minute. After three hours it had slowed down to 12 systoles per minute but was still beating regularly. Now, however, the ventricle was no longer emptying completely and relaxed to three times its previous volume at diastole. By increasing the pressure of perfusion the rate was increased to 18 systoles per minute and, while the ventricle retained the full relaxation at diastole, it returned to complete emptying at systole. After eight hours of perfusion, the rate was down to nine systoles per minute but was restored to eighteen by an increase in pressure. After ten hours of perfusion the rate had dropped again to ten systoles per minute and a further increase in pressure was required to raise it to sixteen. After this ventricle, which had been set aside for three weeks in sea water at 9° C., had maintained an uninterrupted rhythm for fourteen hours and sixteen minutes its threshold for 5HT was found to be in the neighborhood of a 5×10^{-9} molar concentration and its threshold for acetylcholine was 10^{-10} molar.

Of the two hearts set aside for six weeks at 9° C., one survived. When set up in a heart bath and perfused with sea water the ventricle showed no sign of spontaneous activity, but it did react to 10^{-7} molar 5HT by beating at the rate of 18 systoles per minute as long as it was subjected to 5HT.

Subsequently, four hearts were taken which had survived four weeks at 11° C. but which did not beat spontaneously when perfused. Each was subjected to concentrations of acetylcholine and of 5-hydroxytryptamine from 10^{-12} to 10^{-2} molar at half-molar intervals. No concentration of acetylcholine provoked beating. All four hearts beat, when perfused with 10^{-7} molar 5-hydroxytryptamine, at a normal rate but at an amplitude much less than that which had been elicited by 5-hydroxytryptamine after soaking at 9° C. for six weeks.

Discussion

The failure of acetylcholine to restore automatism is in accord with the similar findings of Jensen (1957) with several lamellibranch hearts.

Pharmacology of the radula protractor

Methods

The radula protractor was isolated intact, attached to a bit of radula sac at one end, and to a fragment of odontophore at the other, and set up in a sea water bath at 19° to 21° C. Drugs in sea water solution were added to the bath to produce the desired molar concentration. Ejection from a syringe assured thorough mixing. Air was bubbled through the bath from a capillary tube entering at the bottom.

Results

Spontaneous contractions do not occur in a *Busycon canaliculatum* radula protractor isolated and maintained under slight tension in a constant temperature sea water bath. This makes it a more favorable preparation for the study of induced contractions than the more commonly used snail retractor pharyngis, where rhythmicity obscures the effect of stimulation (Masai, 1951).

Acetylcholine at 10^{-5} molar concentration will induce a contraction which reaches full amplitude immediately. When the acetylcholine is washed off, the muscle relaxes immediately. If the acetylcholine solution is left in contact with the muscle, the contraction declines slightly for five or ten minutes, and then the rate of relaxation accelerates, and by forty-five minutes after adding acetylcholine the muscle is relaxed again. The bathing solution has then lost its ability to contract a fresh muscle. Normal responsiveness of the muscle is restored by ten minutes of washing with aerated sea water.

When 10^{-5} *M* acetylcholine is followed in three minutes by sufficient tryptamine to produce a concentration in the bath of 10^{-3} *M*, relaxation is not an immediate fall in tension such as is seen when acetylcholine is washed off, but is gradual at first and then develops into rhythmic pulsation with a slowly declining base line (Fig. 4, A). The rhythmicity may be as regular in rate and amplitude as a heartbeat and ceases abruptly with the relaxation that follows when the mixture of the two drugs is washed off. In each of the rhythmic contractions the radula protractor shortened to about one-third of its resting length. Figure 4, B, shows the similar response when the muscle is subjected first to 10^{-5} *M* acetylcholine and then in two minutes to the combination of 10^{-5}*M* acetylcholine and 10^{-3} *M* 5-hydroxy-

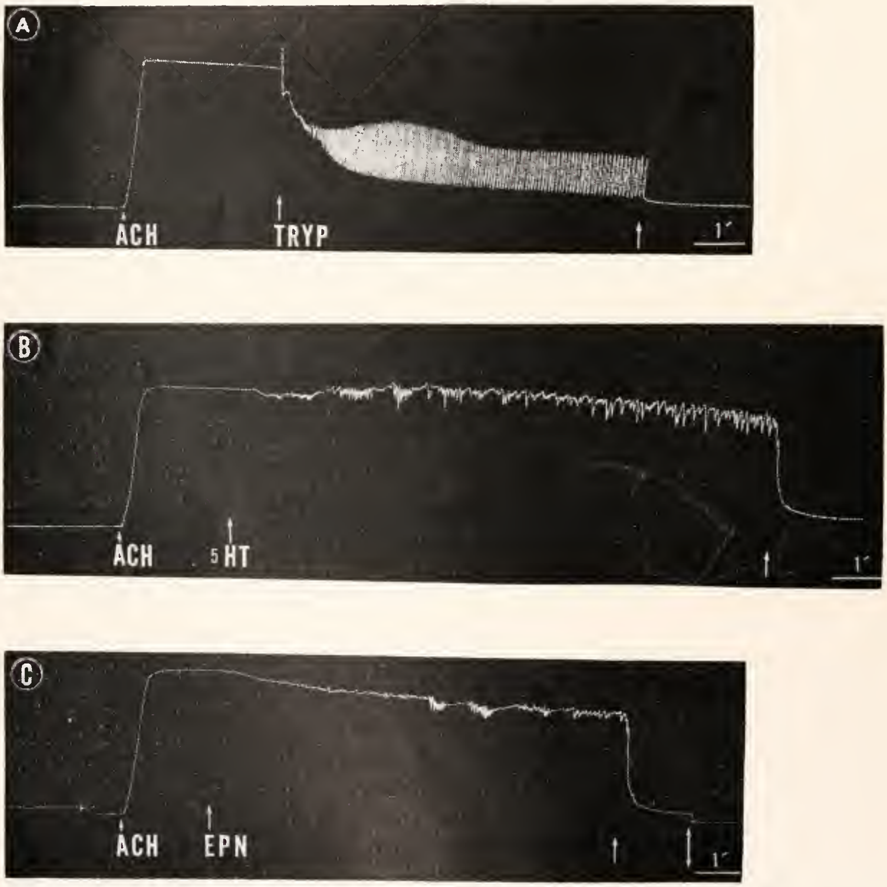

FIGURE 4. Responses of the *Busycon canaliculatum* radula protractor to 10^{-5} molar acetylcholine (ACH) followed by 10^{-3} molar tryptamine (TRYP), 5-hydroxytryptamine (5-HT), or adrenalin (EPN).

tryptamine. Figure 4, C, shows the response obtained when the radula protractor is subjected first to 10^{-5} *M* acetylcholine and then in two minutes to 10^{-3} *M* adrenalin, also. These combinations were found to be optimum for regularity and amplitude of "beat," and it may be seen that tryptamine was the most effective of the three amines.

Figure 5, A, shows that in the radula retractor, tryptamine following acetylcholine produces a similar rhythmic decline of tension. The same is true for the odontophore retractor (Fig. 5, B). It may be seen that in Figure 5, A, the first effect of tryptamine was a slight decline in tension while in Figure 5, B, it was a slight increase. The first effect seems to vary randomly for all three radula apparatus muscles, but is always followed by the rhythmic relaxation.

In Figure 5, A, at T_1, 10^{-5} *M* tryptamine was added in the absence of prior stimulation by acetylcholine, and at T_2, the concentration was brought to 10^{-4} *M* tryptamine. Neither contraction nor relaxation was elicited, yet at the second T_2 the same concentration (following acetylcholine) produced relaxation. At the temperature of these experiments, 19-21° C., no concentration of tryptamine, 5-hydroxytryptamine, or adrenalin relaxed a radula apparatus muscle that had not been previously excited to contract. Later it was found that at 27° C. 5-hydroxytryptamine would cause a previously unstimulated muscle to contract slowly and irregularly, but never to relax.

A radula protractor in 10^{-3} *M* 5-hydroxytryptamine develops a sensitivity to stretching not shown in the muscle simply isolated in sea water. It responds to a sharp tug by a quick contraction followed by slower relaxation.

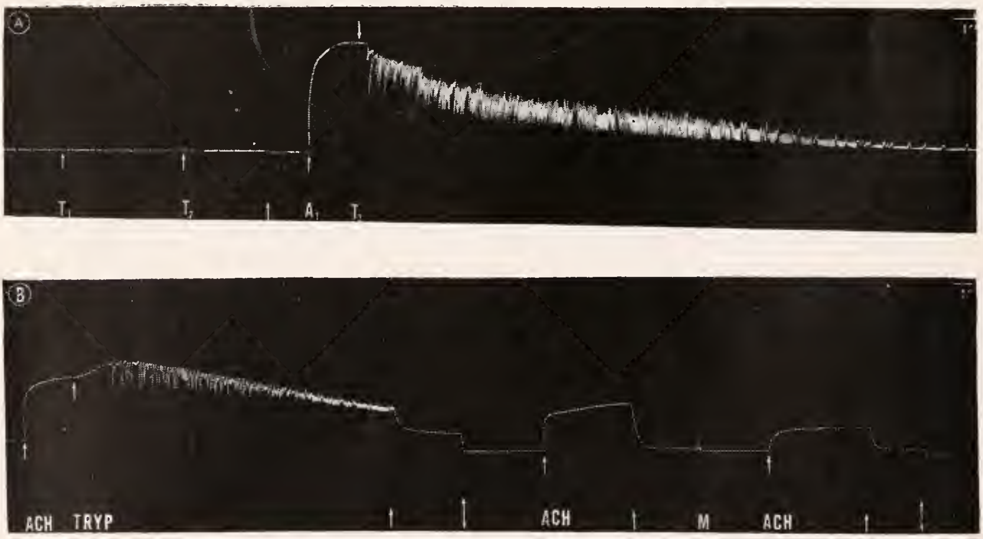

FIGURE 5. *Busycon canaliculatum.* A. Radula retractor: $T_1 = 10^{-5}$ molar tryptamine, $T_2 = 10^{-4}$ molar tryptamine, $A_1 = 10^{-5}$ molar acetylcholine. B. Odontophore retractor: ACH = 10^{-4} molar acetylcholine, TRYP = 10^{-3} molar tryptamine, M = 10^{-5} molar Mytolon for a half hour. A plain arrow indicates that the muscle was washed with sea water with the drum moving, and a double-ended arrow indicates that the muscle was washed with sea water for an hour with the drum stopped.

Acetylcholine contraction of the radula protractor may be blocked both by an agent active at motor end plates, d-tubocurarine, and by the most effective antagonist of acetylcholine on the *Venus mercenaria* heart (Luduena and Brown, 1952), Mytolon. When 10^{-3} molar d-tubocurarine is applied for half an hour the contraction elicited by 10^{-5} molar acetylcholine is blocked but not that due to 0.5% KH_2PO_4. Similarly, 1:10,000 Mytolon applied for a half hour greatly reduces the contraction elicited by 10^{-5} molar acetylcholine but has no effect on the contraction following 0.5% KH_2PO_4.

Acetylcholine contraction of the radula protractor is potentiated by eserine. When the graded responses of the same muscle to an increasing series of acetylcholine concentrations, before and after soaking for an hour in 1:10,000 eserine, are compared, it is found that the response at each concentration is augmented although the threshold to acetylcholine is not altered. Prior soaking in eserine has the same effect on the rhythmicity obtained with acetylcholine and tryptamine as has increasing the concentration of acetylcholine used.

Lysergic acid diethylamide is antagonistic toward the production of a "beat" by the combined action of acetylcholine and tryptamine, but does not itself cause contraction or relaxation at concentrations from 10^{-5} molar to 10^{-10} molar.

DISCUSSION

The rhythmic "beat" of the radula protractor suggests a model of the heart beat. Acetylcholine and 5-hydroxytryptamine both occur as natural neurohumors, both will regulate the *Busycon* heart, and together they induce a rhythmicity in the *Busycon* radula protractor comparable to the automatic rhythmicity of the heart. Tryptamine, however, is more effective than 5-hydroxytryptamine in inducing rhythmicity, whereas 5-hydroxytryptamine is more effective on the heart. Heart strips, when cut to dimensions similar to the radula protractor and set up on the same apparatus, respond to 5-hydroxytryptamine with rhythmic contractions, which are opposed by acetylcholine.

It is tempting to speculate that the radula protractor "beat" might originate in the presence at the cell surface of the opposing neurohumors in the right proportions for alternate action. Welsh and Slocombe (1952) suggest that released acetylcholine depresses the *Venus mercenaria* heart by changing the membrane polarization of muscle fibers and thus interfering with normal contraction and the normal spread of excitation. The effects of acetylcholine and 5-hydroxytryptamine on the surface membrane polarity of a non-cardiac molluscan smooth muscle have been investigated by Twarog (1954). She found that acetylcholine depolarized the *Mytilus edulis* anterior byssus retractor and initiated contraction. 5-Hydroxytryptamine caused immediate relaxation but produced no change in membrane polarization. Furthermore, when the acetylcholine was washed off, the muscle immediately repolarized, but the contraction persisted. (It may be recalled that when acetylcholine was washed off the radula protractor, the muscle relaxed immediately). Twarog suggests that it is probable that the depolarization induced by acetylcholine is directly related to the ensuing contraction. The failure of 5-hydroxytryptamine to produce membrane changes while relaxing the muscle could be attributed to a direct action on the contractile element.

A possible explanation for the rhythmicity induced in the radula protractor by acetylcholine and tryptamine could be based on Twarog's byssus retractor results. It could be supposed that the acetylcholine in the bath kept the muscle cells depolarized, which would lead to contraction. The tryptamine also in the bath would relax the contractile elements and a second contraction would then occur in response to the surface depolarization.

One alternative hypothesis would be that acetylcholine depolarized the muscle fiber surface membrane and that tryptamine then repolarized it. If it is supposed that contraction follows depolarization and relaxation follows repolarization, the "beat" might be explained. That the applied acetylcholine and tryptamine act on muscle rather than on nerve may be indicated by the persistence of susceptibility to induced "beating" in isolated radula protractors stored for a week.

Summary

1. The hearts of *Busycon canaliculatum* and *Strombus gigas* were found to respond to applied neurohumors as do the myogenic hearts of other gastropods. Acetylcholine was cardio-inhibitory, and 5-hydroxytryptamine was cardio-acceleratory, in concentrations low enough to suggest that they might be the normal regulatory neurohumors.

2. The *Busycon canaliculatum* radula protractor was contracted by acetylcholine, and could then be relaxed rhythmically by 5-hydroxytryptamine, tryptamine, and adrenalin, all of which raise the tonus of the ventricle.

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OXYGEN UTILIZATION IN THE SYMBIOSIS OF EMBRYOS OF THE SALAMANDER, AMBYSTOMA MACULATUM AND THE ALGA, OOPHILA AMBLYSTOMATIS

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Unicellular algae occur as symbionts in a great number of aquatic invertebrates, in protozoans, sponges, coelenterates, turbellarians, bryozoans, rotifers, gastropods, lamellibranchs, annelids, and ascidians (Yonge, 1944). An association of unicellular algae and vertebrates has been known only since 1888 when Orr reported a spherical green alga in the egg envelopes of *Ambystoma maculatum* (Shaw). The symbiotic relationship between these salamander eggs and algae was established by Gilbert (1942, 1944) who performed several experiments and made several interesting observations on egg masses of *A. maculatum.*

Masses of these salamander eggs inhabited by the unicellular alga *Oophila amblystomatis* (Lambert) [1] were demonstrated to have lower mortality, an earlier hatching time, and a faster growth rate than algae-free masses: the newly-hatched larvae averaged 1.3 mm. more in length and were more than two Harrison stages in advance of larvae from algae-free egg masses of the same age. Gilbert demonstrated that the results were due to the presence of the algae and not to the presence of light. It was also shown that the algae grow more vigorously in the presence of the embryo than in its absence. Other organisms in addition to the alga inhabit the egg envelopes. These include a protozoan (*Haptophyra* sp.) which feeds on the algae, other protozoa, nematodes, rotifers, acanthocephalans, diatoms (usually on the surface of the egg masses), and even an occasional annelid.

A more rapid growth rate and better viability of embryos from algae-inhabited complexes, as reported by Gilbert, were confirmed during the course of this investigation. These observations suggest that the symbiotic relationship may consist of the utilization by the embryo of the O_2 produced in photosynthesis by the algae and the utilization by the algae of CO_2 produced by the embryo.

Eggs of *Ambystoma maculatum* are often laid in small, temporary, woodland ponds usually containing decaying leaves and other detritus. Such a pond often has a low O_2 content, and any method which would increase the O_2 supply to the embryo might aid in its development. The following experiments were designed to determine if the algae inhabiting the egg membranes produced O_2 in a sufficient quantity to be of value to the developing embryo.

MATERIALS AND METHODS

Eggs of *A. maculatum* in the early stages of development (Harrison Stages 9–13) were collected from a small, detritus-filled, temporary pond in Duke Forest near Durham, North Carolina, on February 26 and 27, 1958. Several days of cold rains and snow preceded the time of collection. The temperature of the water was between 5° and 6° C. at the time the eggs were taken. Algal growth was not evident in these early stages.

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[1] See Gilbert (1942) and Smith (1950) for description of the alga and discussions of correct nomenclature.

The egg masses were separated into approximately equal halves. One half of each mass was then allowed to develop in the light in pond water collected with the eggs; thick algal growths developed in these eggs. The other half was removed from the pond water, placed in spring water, and kept in the dark; no algal growths were visible in these eggs. Egg masses utilized in preliminary experiments were kept at 6° C., but all other eggs used were kept in a constant temperature cabinet at 20 ± 1° C.

All oxygen consumption measurements were made in a Warburg respirometer at 25° C. Slightly different amounts of water were added to the flasks to equalize the volume of their contents. A period of two hours was allowed for temperature equilibration before O_2 consumption was measured. Flasks were not shaken in the experiments reported here.

Light was excluded from the flasks in some of the experiments by wrapping aluminum foil around the flask and manometer stem or by cutting off all lights in the laboratory during hours of darkness. All other determinations were made with approximately 150 foot candles of light at the level of the flasks, measured with a Weston Model 603 Illumination Meter.

Embryos were separated from their surrounding envelopes by placing them in a measuring spoon slightly smaller than the eggs and pressing them gently against the side of a finger bowl, rupturing the membranes and allowing the embryo to drop out when the spoon contents were submerged in the water of the finger bowl. Six to nine eggs, embryos, or envelopes were then placed in each experimental flask.

All measurements in each category of experiments were made in duplicate, reduced to standard temperature and pressure, and expressed as microliters (μl) per hour per embryo, envelope, or a complex of both embryo and envelope. All determinations were made over a period of at least ten hours.

Although the experiments reported here involve, primarily, the algae and embryo, many other inhabitants are present in the egg envelopes. For this reason we refer to the "complex," meaning the entire egg envelopes together with their contents—embryo, algae, protozoa, bacteria, etc.

Availability of materials in the proper stages of development sometimes limited categories of experiments which could be performed. Oxygen consumption determinations were made on the following categories of experimental materials:

I. Algae-free complexes
    1. Stages 12–13
    2. Stages 32–34
II. Algae-inhabited complexes
    1. Stages 12–13
    2. Stages 32–34
        a. Light
        b. Dark

- III. Isolated embryos
  - 1. From algae-free, stages 39–40
  - 2. From algae-inhabited
    - a. Stages 12–13
    - b. Stages 39–40
- IV. Isolated envelopes
  - 1. Algae-free, stages 39–40
  - 2. Algae-inhabited, stages 39–40

RESULTS

Oxygen consumption was linear over the entire periods of observation as seen in Figure 1, where each point represents the average of duplicate flasks in five of the experiments. Little variation was obtained among flasks containing similar experimental material.

Mean rates of O_2 consumption for each category of experiments are shown in Figure 2. Differences in rate of oxygen consumption at different stages must be considered in interpreting these results. According to Hopkins and Handford (1943), the rate of oxygen consumption begins with a low value and rises gradually to stages immediately preceding hatching (Stages 37–38). At this time the rate increases rapidly to a maximum and then falls off during the last few stages of development.

The O_2 consumption of the algae-inhabited complex (Stages 12–13) averaged 1.41 μl and for the complex without algae, 0.97 μl. This represents an increase of 45.4% when algae are present. In later stages (32–34) of development the values were 1.91 μl for the complex with algae and 1.11 μl for the algae-free complex, an increase of 74.8% when algae are present. Thus, the difference between algae-free and algae-inhabited complexes is in the direction of greater consumption by the latter and increases with stages in development of the embryo.

Oxygen consumption of algae-inhabited complexes (Stages 32-34) determined under conditions of darkness averaged 2.50 μl, an increase of 0.56 μl (28.9%) over that of similar complexes in the light. This latter value can be taken to approximate the respiration of the algae in the dark and may be assumed to represent the net amount of O_2 evolved during photosynthesis of the algae, provided the assumption is correct that the respiration of the algae is balanced by O_2 production during photosynthesis. As pointed out below, if the algae are facultative heterotrophs, this may be an incorrect assumption.

Algae-free envelopes consumed 0.84 μl, while envelopes with algae present consumed only 0.41 μl, a difference of 104%. The consumption by envelopes can be accounted for by the respiration of the bacteria, protozoa, etc., living within the envelopes. The difference in O_2 consumption evidently is due to O_2 production during photosynthesis of the algae. This net reduction of O_2 consumption in the envelopes may be of value to the embryo by decreasing the removal of O_2 from the immediate environment. However, there is a net consumption of O_2 by all envelopes, indicating that there is no surplus of photosynthetically produced O_2 available to the embryo. Therefore, a higher O_2 tension could not account for the faster development and greater respiratory rates of embryos associated with algae.

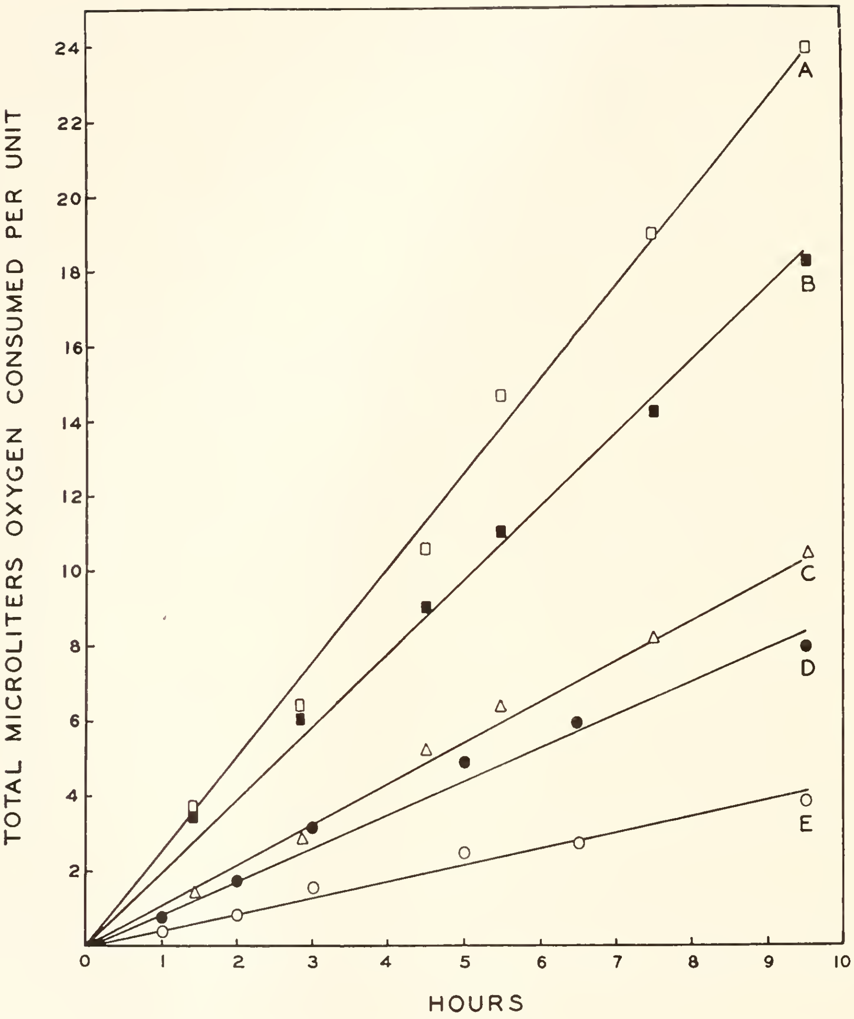

FIGURE 1. Oxygen consumption in five categories of experiments. A, complex with algae in the dark. B, complex with algae in the light. C, complex without algae. D, envelopes without algae. E, envelopes with algae. Embryos in A, B, and C were in stages 32–34.

The difference between O_2 consumption of the isolated envelopes with and without algae in the light was 0.43 μl. This value should approximate the amount of O_2 produced by the algae. If the symbiotic relationship of algae and embryo were only one of respiratory gas exchange, then the consumption of a complex without algae should be greater than a complex with algae, and the subtraction of the amount of O_2 supplied by the algae from the O_2 consumption of an algae-free complex should give a value approximately equal to that of an algae-inhabited complex. The data presented here show that this is not the case. Indeed, such

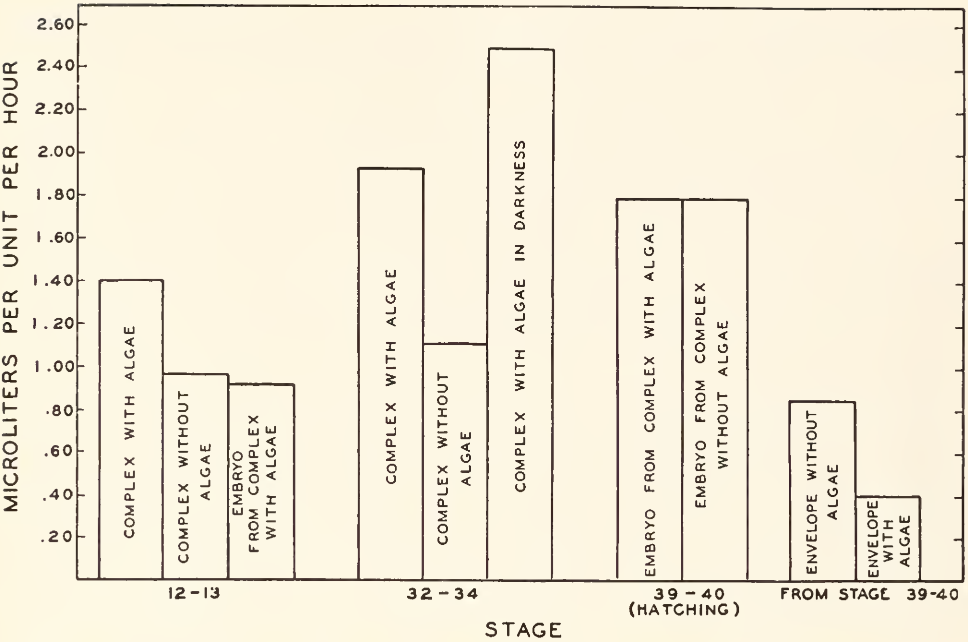

Figure 2. Mean rates of oxygen consumption for each category of experiments.

a subtraction only serves to *increase* the difference in respiration rates of algae-free and algae-inhabited complexes.

Isolated embryos of Stages 12–13 consumed an average of 0.94 μl, practically identical with that of intact complexes without algae, containing embryos in the same stages of development. This suggests that the greater consumption of a complex with algae is due primarily to respiration of the embryos themselves, rather than to inhabitants of the envelopes.

Embryos alone in Stages 39–40 consumed the same amount of O_2, whether from algae-free or from algae-inhabited envelopes. At first glance this would seem to contradict the results of other experiments. The fact that these embryos were in the hatching stage probably lends no support to conclusions about their metabolic rates. Developmental changes during this period are rapid, and activity of individual embryos varies widely. Hopkins and Handford (1943) found that the O_2 consumption of *Ambystoma tigrinum* was consistently higher than that of *A. punctatum* during stages of development up to 41–42. At this stage the variations in results were wide, and species differences were obscured.

Discussion

If photosynthesis by the algae resulted in a supply of O_2 for the embryo, it is reasonable to assume, given equal requirements by the embryos, that the net O_2 consumption of algae-inhabited egg masses in the light would be less than the consumption of algae-free complexes. However, the opposite results were obtained.

The results given above indicate that embryos with envelopes containing algae respire at a greater rate than embryos in the same stage of development without the benefit of algae. Furthermore, this increase in O_2 demand is not equaled by the smaller amounts of O_2 evolved by the algae during photosynthesis. These results lead to the conclusion that the symbiotic relationship of embryos and algae is not one of respiratory gas exchange alone, and imply some other factor—possibly a growth-stimulating substance, which would account for higher respiratory rates, faster growth, better survival, and larger size at hatching. The fact that a complex with algae consumes more O_2 (and linearly) in the dark than a similar complex in the light as compared with an algae-free complex, indicates that this growth factor is probably supplied in equal amounts during photosynthesis and in darkness, since the rate of consumption continues at the same high level throughout the ten hours of measurements.

The isolation and identification of such a growth factor might require the use of extracts from the algae grown in pure culture. The fact that the alga involved inhabits only *Ambystoma* egg envelopes (with one exception, where it was found in *Rana sylvatica* envelopes) suggests that the determination of a proper culture medium would not be easily accomplished. If such culture media were developed, the different components of algal extracts added to eggs without algae might lead to the proper identification of the substances involved. The presence of numerous bacteria within the envelopes indicates that the factor involved is probably not an antibiotic.

Several problems are suggested by the presence and better growth of the algae associated with embryos. It is likely that the algae obtain organic nitrogenous substances from the embryo. The observation by Gilbert (1942) that the alga is thickest in the inner envelope and is especially numerous around the proctodaeum of the embryo suggests that nitrogenous wastes or bicarbonates are being utilized by the algae. Experiments by Gregg and Ballentine (1946) indicate the presence of CO_2 as bicarbonate in the blastocoel and archenteron fluid of amphibian embryos. If the alga is a facultative heterotroph, then it is inaccurate to assume that its photosynthesis balances or exceeds its respiration. In addition, the alga is unknown from outside egg masses, and the process used to migrate into and through the gelatinous egg coverings is also unknown.

Buchsbaum (1937) has shown that a metabolic mutualism occurs when a culture of the green alga *Chlorella* is combined with embryonic chick tissue cells, where both were favored over algae alone or chick tissues alone. He found that tissues without algae grew better in the dark than those with algae and concluded that the results were "best explained on the basis of a photosynthetic mutualism." These conclusions were based on measurements of growth alone and not on differences in respiratory rates.

Buchsbaum's data suggest that the mutual benefits derived from an association of *Chlorella* and chick tissue cells are due to gas exchanges involved in photosynthesis, or if some other factors are responsible, they are produced only during photosynthesis. Our results indicate the opposite—that photosynthesis alone cannot account for all of the increased respiration and growth of the embryos, but that other factors are involved in the facultative mutualism of *Ambystoma* embryos and the alga *Oophila*.

We wish to express our appreciation to Dr. H. J. Humm, Dr. I. E. Gray, and Dr. J. R. Gregg for helpful suggestions and critical reading of the manuscript.

SUMMARY

1. Oxygen consumption measurements were made on embryos of the salamander, *Ambystoma maculatum,* which develop within a gelatinous envelope usually inhabited by a unicellular green alga, *Oophila amblystomatis,* and numerous protozoa and bacteria.

2. A comparison of embryos associated with the alga, and others of the same developmental stage but lacking visible signs of the alga, reveal that the former respire at a greater rate in both early and later stages.

3. In darkness the oxygen consumption of the algae-containing complexes is greater than in the light, suggesting that photosynthesis by the algae answers a portion of the oxygen demand. This is also indicated by the fact that isolated green envelopes consume only half as much oxygen as isolated colorless envelopes. Net consumption by the isolated green envelopes is taken to mean that algal oxygen production is not sufficient to meet the requirements of the heterotrophic inhabitants of the envelope, and therefore could not provide a surplus to the embryo. It is suggested that the higher metabolic rates and more rapid development of embryos associated with algae must depend in part on some factor other than oxygen supplied by photosynthesis.

4. The problem of proving the existence of a growth factor is pointed out, and some additional unsolved problems regarding the life of the alga and its means of penetration into the egg envelope are indicated.

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A COMPARISON BETWEEN TASTE RECEPTORS AND OTHER NERVE TISSUES OF THE COCKROACH IN THEIR RESPONSES TO GUSTATORY STIMULI[1]

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It has long been known from behavioral studies that some butterflies, flies, and bees have taste receptors on the tarsi (Minnich, 1921, 1929, 1932). The electrophysiological studies reported here were begun to determine whether or not such tarsal taste receptors are present in the American cockroach, *Periplaneta americana*. In the course of these studies a clue was found to a fundamental characteristic of all taste perception in the cockroach. This is the similarity between the responses from recognized taste receptors and from nerve tissue unspecialized for taste perception. It is this relationship, briefly described earlier (Roys, 1956), which is the principal subject of this paper.

MATERIALS

Adult male and female American cockroaches, *Periplaneta americana*, of various ages were used for all the electrophysiological experiments, while the behavioral experiments included nymphs of both sexes as well. Sex differences did not seem to have any effect on the responses in the experiments. They were all kept at room temperature and fed on powdered dog biscuit (Purina chow).

The chemicals used in the experiment were all of reagent grade except for the quinine which was U. S. P.

Nerve action potentials were picked up from the tarsal preparations through tungsten electrodes drawn to fine points in a gas-oxygen flame. The electrodes were connected by copper leads to a Grass P-3A amplifier and a Dumont 208B oscilloscope. A Grass cathode follower was also used in some of the experiments. In nerve cord preparations, bare silver electrodes were substituted for tungsten. Changes in nerve activity were measured with an Electrodyne decade impulse counter which recorded the number of nerve impulses per second. This was connected to the output of the oscilloscope amplifier.

EXPERIMENTS AND RESULTS

Seven types of experiments were carried out—six on the response of various types of nerve preparations to a test substance and one on the behavioral response to the same substance presented in the drinking water. Four test substances were used—sodium chloride, hydrochloric acid, sucrose and quinine, corresponding to the four accepted taste sensations of salt, sour, sweet and bitter. First, sodium

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[1] This work was made possible by a grant from the U. S. Public Health Service to Tufts University. Some of the apparatus used was obtained under a previous contract between the U. S. Chemical Corps and Tufts University.

chloride was used in all seven types of experiments, then hydrochloric acid, sucrose and quinine, making a total of 28 different experiments. Each of these 28 was repeated at least five times to insure the validity of the results. In the following sections A through G, the rationale, techniques and results of the experiments with sodium chloride are discussed in some detail. Section H deals with substitution of hydrochloric acid, sucrose and quinine for sodium chloride in these same experiments.

A. Intact tarsus

To test whether application of sodium chloride solutions to the tarsus would produce any afferent activity in the nerve of the leg, a prothoracic leg was cut off at the femoro-tibial joint and mounted with two electrodes in contact with the nerve. One was inserted into the opening at the cut end of the leg, the other pushed in through the membrane at the tibio-tarsal joint until the tips were about one millimeter apart within the tibia. The electrodes supported the leg, and the tarsus extended down into a wax cup filled with water or test solution which could be changed with a pipette. Because of the large diameter of the electrodes relative to the size of the tibia, they usually detected afferent impulses without special care as to their exact position. The tungsten electrodes were connected to the amplifier and oscilloscope.

When the tarsus was submerged in water in the wax cup, the nerve showed a steady discharge of typical nerve spikes which probably originated in mechanoreceptors of the leg. This basal activity was measured by counting the number of spikes per second with an electronic counter set to count all spikes above the noise level. Ten consecutive counts were taken in a group and averaged. When two or more successive groups of counts showed the same level of activity, it was considered that a satisfactory base had been established. Then the water was replaced with sodium chloride in successively higher concentrations of 1, 2, 3, 4 and 5 *M*. Each concentration was left in contact with the tarsus for approximately one minute. The nerve activity continued at about the same level until a sudden increase showed that the threshold had been reached, *i.e.*, that tarsal stimulation occurred at that concentration.

Selection of the "threshold" response must, from the nature of the experiments, be somewhat arbitrary, since the basal activity showed continual small fluctuations. To be certain that the threshold was a valid one, a point was selected where the activity clearly exceeded any of the preceding base line fluctuations or any probable instrumental changes caused by shifts in line voltage, etc. Usually when a clear increase of 100% or more in the number of spikes per second occurred immediately after a change in concentration of the chemical under test and lasted longer than the quick-adapting tactile response, it was considered to be the threshold point. In some instances lower percentage increases were accepted as thresholds due to special circumstances such as an unusually even base line. Experiments were continued until five or more such thresholds at the lower end of the range coincided within half a log unit of one another. The range of the five is given in Table I and the lowest one is plotted as the threshold indicated by the bar graph in Figure 1-A.

The threshold was usually reached at 5 *M* sodium chloride. However, it was variable and in one instance occurred at a concentration as low as 1 *M*. The roaches used in this series of experiments were all adult females, but their age as adults was unknown. If the age was the variable factor, then it seemed possible that the work of Slifer (1950) on locusts offered a further explanation of this variation in thresholds. She found that water permeability of the cuticle on locust tarsi increased with age because the impermeable outer layer was abraded away in older individuals. This suggested that permeability of the tarsal cuticle might be the

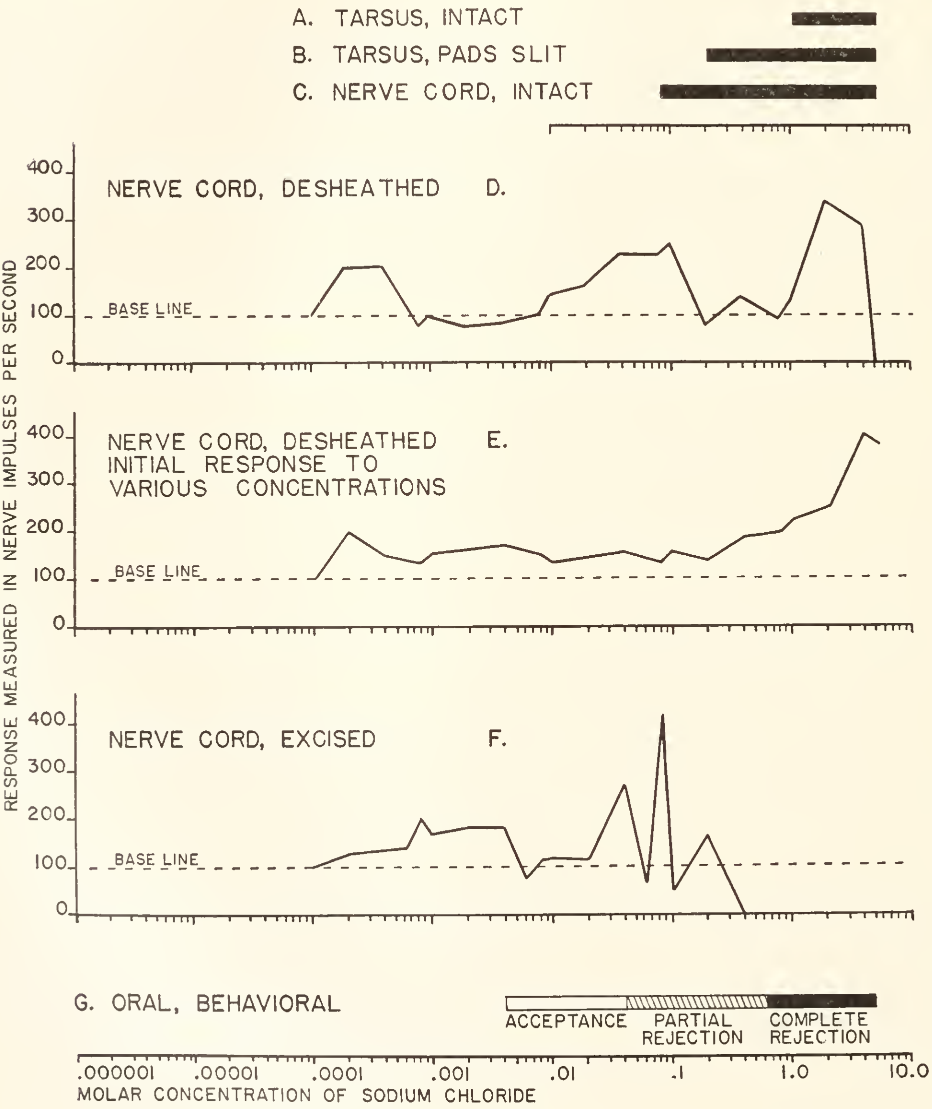

FIGURE 1. Responses of all types of preparations to sodium chloride

TABLE I

Thresholds for the four major taste qualities in different types of preparation

	Thresholds						Calculated osmotic pressure at slit tarsus thresholds
	Tarsus intact	Tarsus pads slit	Nerve cord intact	Nerve cord desheathed	Nerve cord excised	Behavioral oral	
Salt Sodium chloride	1–5 M	0.2–0.4 M	0.08–0.1 M	0.0002–0.0006 M	0.0002–0.0006 M	0.004–0.008 M	9.76 Atm.
Sour Hydrochloric acid	0.1–0.6	0.006–0.008	0.001–0.004	0.0002–0.0004	0.00001–0.00004	0.0006–0.0008	.29
Sweet Sucrose	No response at 2.5 M	0.1–0.4	0.1–0.6	0.002–0.004	0.001–0.004	0.006	2.44
Bitter Quinine	No response at 0.01 M	0.001–0.008	0.001–0.006	0.00008–0.0001	0.000006–0.000008	0.0002–0.0004	.02

factor controlling the tarsal thresholds in cockroaches. If so, slitting the tarsal pads to allow free entry of the test solution could be expected to lower the threshold.

B. Tarsus with pads slit

The tests were repeated using tarsi the five tarsal pads of which had been slit longitudinally with a razor blade. Concentrations for these experiments were increased by steps in accord with the following series: .01, .02, .04, .06, .08, .1, .2, .4, .6, .8, 1.0, etc. This progression of concentrations was also used for most subsequent experiments, but in some it was shortened to .02, .04, .08, .2, .4, .8, 2.0, etc., which approximates a doubling of concentration at each step. Because the test solutions would enter the tissue, they were made up in saline solution (9.0 g. NaCl, 0.2 g. KCl, 0.2 g. $CaCl_2$ per liter of solution; proportions from Pringle, 1938) instead of water. Thus in the sodium chloride of the test solution was included the amount of sodium chloride in saline solution plus the sodium chloride added. For example, at a threshold of 0.2 M sodium chloride in saline solution, the sodium chloride present was 0.15 M (from 9.0 g. per liter in the saline solution) plus the additional 0.2 M, giving a total concentration of 0.35 M. The same criteria for thresholds were used as for intact tarsi in the preceding series. The results are shown in Table I and Figure 1-B and it can be seen that the thresholds are much lower than for the normal intact tarsi.

C. Nerve cord in situ, intact

However, when the test solution entered the tissue of the tarsus it reached all types of nerve endings. Did the afferent response originate in special subcuticular taste receptors or in nerve endings unspecialized for taste? One way to answer this question was to compare the responses of the tarsi to those from a nerve located where it could not normally be concerned with taste perception. Such nerves are found in the connectives and ganglia of the ventral nerve cord. If they respond to the same concentrations of sodium chloride it would indicate that the response of the tarsi was not dependent on special taste receptors.

It was found to be possible to pick up impulses from the ventral nerve cord in the intact roach simply by slipping the active electrode between the sternal plates and into the abdominal cavity just ventral to the abdominal cord. For routine experiments the roach was anesthetized with carbon dioxide, laid on its back on a small lucite block and secured in place with strips of Cenco Tackiwax. A bare tungsten electrode was inserted between the second and third abdominal sternites. The indifferent electrode was inserted in the opening formed by cutting off the tip of an antenna. Since the roach antenna contains no muscles or efferent nerves, an electrode placed there does not pick up any extraneous nerve or muscle potentials. The test solution was injected close to the cord and posterior to the pickup electrode with a fine glass pipette. Base line and threshold were established as before and the thresholds obtained are shown in Table I and Figure 1-C. They are slightly lower than those from the tarsi, indicating that the tarsal response is not entirely dependent on special taste receptors.

D. Nerve cord in situ, desheathed

Twarog and Roeder (1956) showed that the sensitivity of the roach nerve cord to chemicals was greatly increased when the connective tissue sheath was removed. To test the effect of this sheath on the sodium chloride threshold, the experiments were repeated using a modification of the Twarog window preparation. For this preparation the head and hind legs of the roach were removed, and the roach laid on its back on a plastic block and held in place by a band of Tackiwax across the thorax, leaving the ventral side of the abdomen exposed. With a fine pair of scissors two windows were cut in the ventral abdominal wall. For one, the central section of the second sternite was cut away to expose the ventral nerve cord. A loop of the exposed cord was lifted out and hung over a bare silver wire which formed the pickup electrode. As soon as the nerve cord had dried enough to stick to the wire a little, it was cut on the anterior side of the electrode to limit activity to that originating in the abdomen. When a cathode follower was used with this type of preparation it was possible to let the exposed section of nerve cord dry completely, the pickup then being through the dry dead section to the living cord inside the body (Roeder and Treat, 1957). The indifferent electrode was inserted into the body cavity at the neck. The second window was formed by cutting away the central portion of the fourth, fifth and sixth sternites to expose a nerve cord ganglion and its adjacent connectives. Under this section of cord was placed a narrow strip of Parafilm or wax paper to separate it from the underlying tissue and body fluid. The connective tissue sheath was torn with fine forceps (see Twarog and Roeder, 1956) and the exposed section perfused with saline followed by a series of test solutions. The perfusing fluid was supplied from a small reservoir through a fine glass tube and carried away by a wick of absorbent paper.

The results are shown in Table I and Figure 1-D. The base line of this and all subsequent curves is adjusted to 100 for convenient comparison with other experiments. Curve D is taken from a single experiment considered typical of those done. The salient peaks and hollows were found in curves from all the experiments of the series, but they varied in their exact position on the x-axis so that an addition of several curves to form a composite would conceal the true form of the curve through cancellation. Hence, the single representative curve is given instead of a composite based on several experiments.

It will be noted that the thresholds are much lower than those of either intact sheathed cord or tarsus, clearly showing that the sheath checks penetration of sodium chloride and conversely that activity stimulated by these low concentrations must come from within that part of the cord normally enclosed by the sheath.

E. Nerve cord in situ, desheathed. Initial response to various concentrations

The preceding curve D, showing the response to steadily increasing concentrations of sodium chloride, has a characteristic succession of peaks and depressions. On first thought, one might interpret this to mean that the nerve does not respond to certain concentrations of sodium chloride, *e.g.*, between .0008 *M* and .008 *M*. However, it seemed more likely that these depressions represented some sort of adaptation to continued exposure to sodium chloride, and that a fresh nerve cord would respond initially to any concentration of sodium chloride above the threshold value of .0002 *M*. To check this a series of experiments was run to determine the response of a freshly dissected nerve cord in saline to each concentration shown on the curve. The results are shown in Figure 1-E. At the highest concentrations, blocking of all nerve activity began before the ten counts were completed so that the end of the curve, based on an average of ten readings, shows a drop even though the first one or two readings are higher than any preceding ones.

It can be seen from the figure that there are no concentrations above threshold to which the nerve does not initially respond, and that the extent of response generally increases with the concentration of sodium choride, particularly at high concentrations. This seems to support the inference that the depressions in curve D are due to the cumulative affect of previous treatment and do not result solely from the concentrations at which they appear.

F. Excised nerve cord

While the experiments with the normal and desheathed cord showed a clear response to rather low concentrations of sodium chloride, it may be questioned whether this response was due to direct action on the nerve cord. Possibly the perfusing fluid leaked down onto some unsuspected area of taste receptors. To check this possibility the abdominal section of the nerve cord was removed from the roach and tested alone. This consists of a chain of six ganglia joined by paired connectives and is about one centimeter long. It was dissected out and laid across silver wire electrodes in a small depression in a lucite block, in a modification of the preparation described by Roeder and Roeder (1939). When this depression was filled with saline or test solution, the cord was submerged, and no impulses were picked up because of electrical shunting through the saline solution. However, when the solution was drawn out of the depression with a small piece of absorbent paper, the moist cord was left hanging in the air across the two electrodes and impulses were picked up for viewing on the oscilloscope and for counting.

To establish the base line activity of the cord in saline solution, it was first submerged in saline for several minutes to equilibrate with the new medium, then the solution was drawn off, ten counts of the number of spikes per second taken, and the cord submerged in saline again for one minute before another count was taken. As soon as the base level of activity had been established as for the intact tarsus, test solutions of increasing concentrations of sodium chloride were substituted for pure saline. Thus the cord was alternately submerged and exposed for periods of one minute each during the experiments, in contrast to the preceding experiments where the nerve was continually perfused. However, length of exposure to each test solution was approximately the same in both types of experiment.

Although the excised nerve cord was not desheathed, numerous openings were left wherever connectives were cut away and where the whole cord was cut at the anterior end in the process of excision. Therefore, it seems probable that the protective function of the sheath was reduced almost as much as by stripping it away.

In this preparation all six ganglia and their connectives were simultaneously exposed to each change of solution, in contrast to the preparations with the nerve cord *in situ* where only a single ganglion and its connectives were exposed. This produced responses in the excised cord which were more sharply defined and the thresholds were more easily determined than with the cord *in situ*. It also resulted in blocking at a lower concentration than that which blocked the cord *in situ*, where some of the ganglia and connectives were protected from direct exposure to the salt.

From Table I and Figure 1-F it is clear that the threshold is at least as low as that of the cord *in situ*, further confirming that no special taste receptors are needed to account for the response. Considering the preceding six types of experiment in retrospect, it is now clear that as the protective coverings are stripped away—first the cuticle to expose the leg nerve and intact cord to chemical action, then the sheath from the exposed cord—the sensitivity increases. Receptors specialized for taste are usually assumed to be more sensitive to chemicals than other nerves. However, in view of the low threshold of the desheathed nerve cord it seemed worth while to check the sensitivity of recognized taste receptors for comparison.

G. *Oral taste, behavioral*

Evidence from various sources and confirmed by Frings and Frings (1949) indicates that the maxillary and labial palpi of the cockroach carry the oral taste receptors. Therefore the palpi were set up in the same way as the tarsal preparations, in the expectation of getting responses in line with the behavioral thresholds reported by Frings (1946). However, the thresholds obtained were only slightly lower than those from the intact tarsi. It seems probable that this discrepancy was due to the very small size of the fibers which carry the normal gustatory responses —a condition which makes recording very difficult. To avoid this difficulty the method developed by Hodgson, Lettvin and Roeder (1955) was tried. Working with flies they were able to make electrical contact with single sensory hairs in the oral region which responded clearly to chemical stimuli. However, this method proved unsuccessful when applied to the cockroach. The longer hairs at the tips of the palpi did not give any clear responses to gustatory stimuli, and it may be that gustatory perception is through short bristles or pegs which lie between the longer hairs and are therefore rather difficult to reach with the electrode. Therefore, for the present we must rely on the older method of behavioral response.

Behavioral thresholds for sodium chloride were determined by a modification of the method used by Dethier and Rhoades in 1954 for flies. This method offers the test population a choice between flavored and unflavored drinking water and measures the amount of each kind consumed in each of a succession of trial periods. In each trial the concentration of flavored material is greater than in the preceding one. Any change from a one-to-one ratio of consumption indicates ability to distinguish between the two, *i.e.,* the threshold concentration of the flavoring material.

In the first of these experiments a colony of about 100 adult roaches of both sexes was used for one series of experiments and a colony of nymphs for another series. However, no differences between the responses of nymphs and adults were noted and in subsequent experiments a breeding colony of mixed nymphs and adults of both sexes was used. The roaches were confined in 15-gallon aquaria containing cardboard shelters, but were not restricted in any other way and were free to eat and drink whenever they chose. Temperature ranged from 22° to 26° C. and the cages were lighted during the day by room illumination and were dark at night. This near-normal environment and lack of any restriction to movement of the experimental animal are notable advantages of this method.

The drinking water for each colony was supplied from two identical glass tubes of 6 mm. inside diameter and about 50 cm. long. These tubes lay parallel on the bottom of the aquarium except for the ends which were bent up to prevent the water from running out. At one end the bent sections came up at right angles for 12 cm. and were taped to the wall of the aquarium to hold them in position. The other two ends sloped up at 30° to a height of 1.5 cm. above the aquarium floor and were plugged with rolled cylinders of lens paper which acted as wicks to draw the water from the long horizontal reservoirs and make it available to the roaches. It was found best to put the ends of the tubes with the wicks about one centimeter apart, fastening them to a spacer block with Tackiwax to hold them firm. They were in an open area of the aquarium floor and in the light. The lens paper plugs were changed daily. Each day each tube was filled with water from a graduated syringe to a mark 1.5 cm. up on the vertical section. Thus the amount of water consumed in the preceding 24 hours was determined by measuring the amount needed to refill the tube to the mark. A colony of 100 roaches took about 10 ml. of water a day or 5 ml. from each tube if the tubes were equally preferred. Evaporation, checked in a separate tube, was nearly constant at 0.5 ml. per day. Day-to-day fluctuations in consumption from the two tubes were erratic and ran as high as 20% difference between the two with water in both tubes. To determine the salt threshold, increasing concentrations of sodium chloride (in the same steps used in the nerve preparations) were substituted for water in one of the tubes. The threshold concentration was clearly marked by a sharp increase in preference for the tube containing sodium chloride, *i.e.*, an acceptance threshold.

This was followed by a continued preference for the sodium chloride tube at higher concentrations until the rejection threshold was reached when there was a sharp change in preference from sodium chloride to water. Higher concentrations of salt were progressively less and less acceptable until a concentration was reached at which no salt solution at all was taken, *i.e.*, salt was completely rejected at or above that concentration. Supplementary experiments showed that previous conditioning had little effect on any given trial. For example, in a given pair of tubes when an unacceptable sodium chloride solution was replaced by water, there was an immediate return to an approximately one-to-one ratio of preference and the same appeared to be true in shifting from an acceptable solution to water.

Two complete series, ranging from well below the acceptance threshold to well above the point of complete rejection, were run. In addition three short series were run in the ranges of the acceptance and rejection thresholds. The results are shown in Table I and the bar graph in Figure 1-G, and it is clear from these that the behavioral threshold is well above that of any of the nerve cord preparations.

H. Responses from all types of preparation to sour, sweet and bitter stimuli

To test whether comparable thresholds and curves could also be obtained from sour, sweet and bitter substances, similar series of tests were carried out with hydrochloric acid, sucrose and quinine. Quinine monohydrochloride was substituted for quinine at concentrations above 0.001 *M* because of its greater solubility. At lower concentration it had the same threshold as the pure alkaloid. The experiments with sucrose and quinine were not carried out to 5.0 *M* because sucrose solution becomes a thick syrup above 2.0 *M* concentration, while quinine monohydrochloride tends to precipitate out of solution at concentrations much above 0.01 *M* in water. In every other way the same procedure was followed as for sodium chloride.

The results of these experiments are shown in Table I and Figures 2, 3 and 4. Figure 5 is a summary of all the thresholds for all four substances, plotted on a single graph for comparison.

In comparing the responses to hydrochloric acid with those to sodium chloride it is at once apparent that most of the hydrochloric acid thresholds are lower. An apparent exception is found in the thresholds of the desheathed nerve cord *in situ*. This discrepancy is probably due to a limitation of technique rather than a real physiological difference. This technical limitation lies in the fact that much less area is exposed to test solutions with the cord *in situ* than with it excised (see section F). For this reason the responses from the cord *in situ* were less clearly defined and the threshold less easily determined. In the cases of sodium chloride and sucrose the experimental evidence seemed to support thresholds of the cord *in situ* as low as those of the excised cord. With hydrochloric acid and quinine the evidence at hand did not seem to warrant a firm statement to this effect, although there is a strong probability that it is true for these substances also.

As with sodium chloride, the behavioral tests with hydrochloric acid showed both acceptance and rejection thresholds, and concentrations causing rejection also stimulated the tarsi and produced a strong response in the nerve cord.

Sucrose did not stimulate the intact tarsus in concentrations up to 2.0 *M*, and thresholds for all types of preparation were rather high. In the behavioral tests sucrose was acceptable at all concentrations from threshold to 2.0 *M*.

Quinine also failed to stimulate the intact tarsi, suggesting that the cuticle is less permeable to the large molecules of sucrose and quinine than to the ionized salts and acids. In behavioral tests quinine was rejected in all concentrations above the threshold.

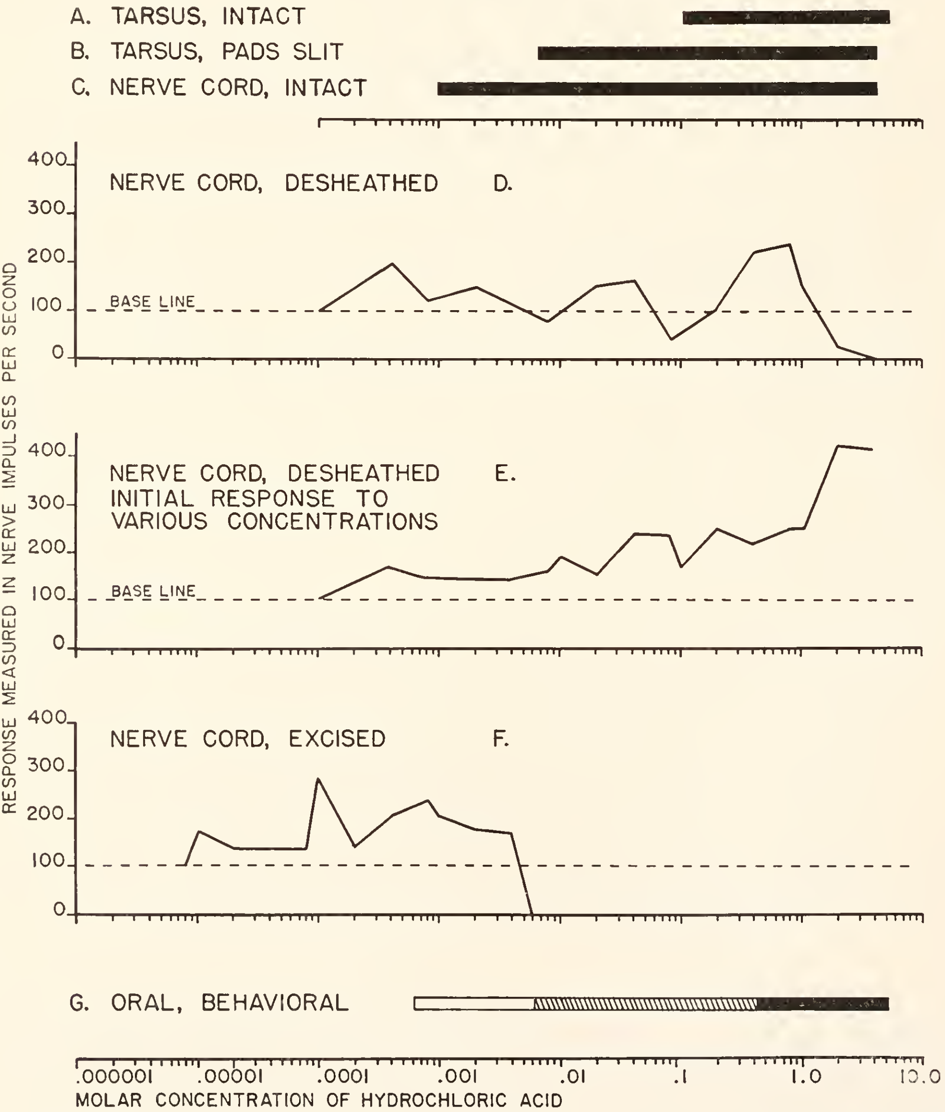

FIGURE 2. Responses of all types of preparations to hydrochloric acid. Legend for bar: unshaded area, acceptance; cross-hatched area, partial rejection; black area, complete rejection.

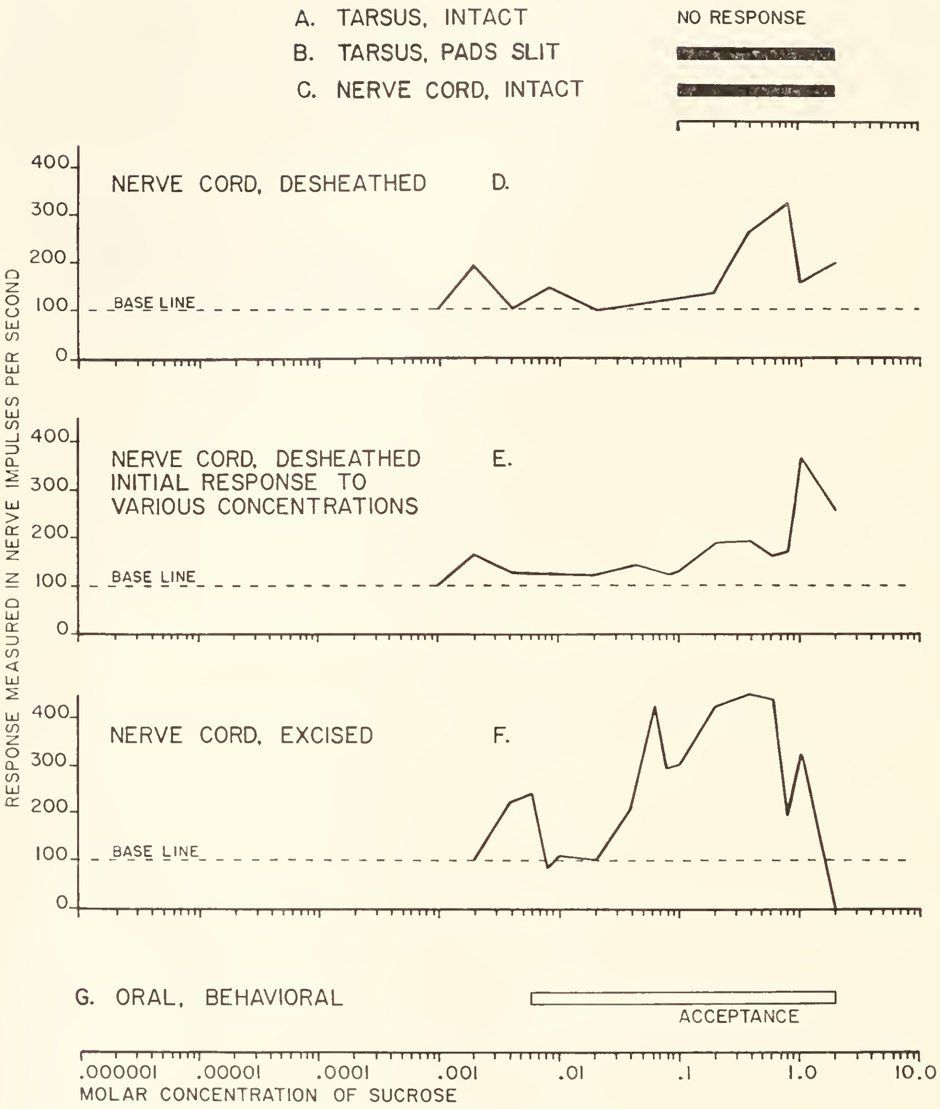

FIGURE 3. Responses of all types of preparations to sucrose.

DISCUSSION

The foregoing sets of experiments seem to indicate that the chemical sensitivity of recognized oral taste receptors, as well as receptors on the tarsi, is always less than that of many other nerves in the body not normally concerned with taste. However, there are a number of points in the work which may need clarification or can profitably be amplified.

The first consideration is whether the nerve responses obtained are true chem-

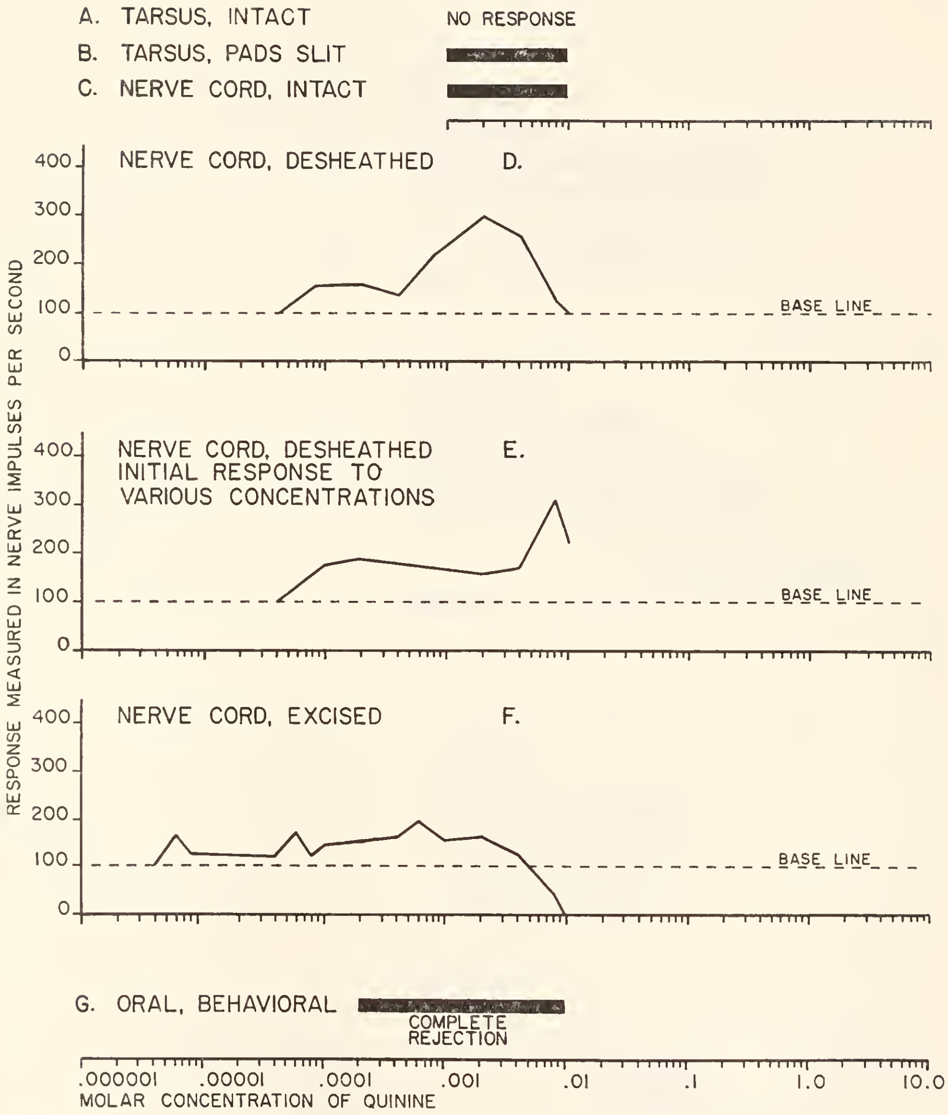

FIGURE 4. Responses of all types of preparations to quinine.

ical thresholds or whether they are due to osmotic or other physical changes. If they are due simply to increased osmotic pressure, then, conversely, threshold concentrations of all four substances should produce the same osmotic pressure. However, the computed osmotic pressures at threshold concentrations for sodium chloride, hydrochloric acid, sucrose and quinine are given in Table I and show a wide range of values for the four substances. This would seem to show conclusively that osmotic pressure was not the principal cause of the responses. The wide range

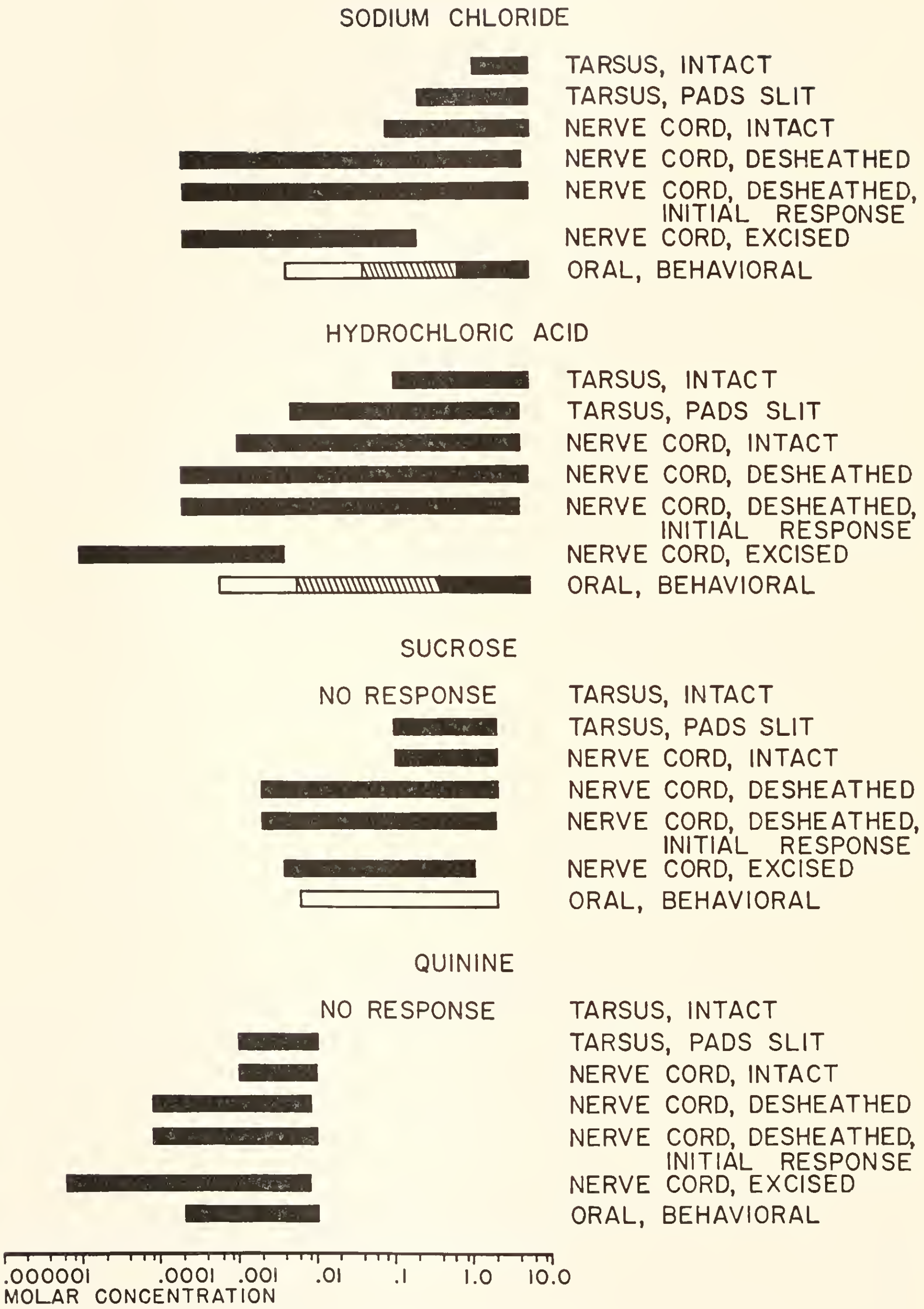

FIGURE 5. Responses of all types of preparations to sodium chloride, hydrochloric acid, sucrose and quinine

over which the thresholds extend seems to be further evidence against a single physical factor being responsible.

Secondly, some may question the physiological validity of certain of these thresholds. In particular the response of the nerves to sucrose may seem to be in direct conflict with the widespread practice among neurophysiologists of using "inert" sucrose to maintain the osmotic pressure of physiological saline solution in the absence of certain salts. However, it should be emphasized that at threshold concentrations the effects of these solutions are transitory. It is customary to allow a short period of time for any nerve preparation to recover from injuries of surgery, small osmotic changes produced when saline solution is substituted for blood, etc., before the activity is studied. Thus these transitory threshold responses might easily be overlooked or masked by other adaptive changes in activity. However, they show up clearly when separated experimentally from other possible causes of nerve activity. Moreover, when used in adjusting osmotic pressure the sucrose is substituted for some constituent of the solution, not added to the solution as was done in these experiments.

It is also possible that injury from operative techniques may have made the nerves more sensitive to chemical action. This seems unlikely in the tarsal preparations where injury was minimal and in the normal nerve cord where test substances were injected into an otherwise intact animal. Also, the Twarog window preparation was particularly designed to do a minimal amount of damage, and this is supported by the fact that thresholds for nerve cord exposed in the window with the sheath intact were no lower than those obtained by injection of the same substance. Desheathing may have torn some of the nerve fibers, but it seems very unlikely that these could have amounted to more than a small fraction of the total which responded to threshold concentrations. However, in the excised nerve cord a large number of connecting fibers were cut to remove it and these cut ends may have been very sensitive. This may have been a factor in the very low thresholds obtained from this type of preparation in response to stimulation by hydrochloric acid and quinine. If so, this would be interesting in itself, but these two thresholds are not essential to the general thesis of this paper.

Another important consideration is whether or not the thresholds from the intact tarsi and from exposed nerves are truly comparable to oral taste. The strongest argument in favor of this is the close correlation between the behavioral threshold and the thresholds of the various nerve preparations in response to a given chemical. This is most clearly shown in the responses to sucrose and quinine (see Figure 5) where the thresholds of the various nerve preparations for quinine are much closer to the behavioral threshold for quinine than to the behavioral threshold for sucrose and vice versa. The same may be said for responses to any other pair of test chemicals though it is more conspicuous in some pairs than in others.

Further correlation is found between the behavioral rejection thresholds for sodium chloride and hydrochloric acid and the activity produced in the nerve preparations by the same concentrations of the salt or acid. These concentrations suffice to raise the activity in desheathed nerves to a very high level and initiate activity in those nerves protected by a sheath or even by a sheath plus cuticle. This correlation seems to lend additional support to the relation between behavioral responses and the responses from nerve preparations. Further, it suggests that when the stimulating chemical reaches a concentration which produces violent activity in the special taste receptor and begins to produce activity in all other nerves in the area, behavioral rejection sets in.

Results from the experiments with quinine do not show this correlation as clearly, while with sucrose we find violent activity in the nerve cord correlated with behavioral acceptance. However, this need not detract from the significance of the correlation between behavioral rejection and nerve response with salts and acids, since it is probable that the bitter and sweet molecules act on the receptors through quite different mechanisms than the salt and acid ions.

The protection afforded by the sheath in these cases brings us to a general summary of the factors which determine thresholds in different types of preparation. In the leg nerve preparation the tarsal cuticle was ruptured with the result that a lower concentration of chemical was needed to initiate nerve action. However, the sheath remained as a second line of protection. It was not practical to remove the sheath from the leg nerve, but a switch to the nerve cord showed similar thresholds. When the nerve cord sheath was removed, the concentration required for stimulation again dropped markedly. Viewing these experiments in general terms, it now seems probable that the *true* nerve threshold is the concentration of test substance which, when acting directly on the unprotected nerve, will produce a response. Any higher thresholds for protected nerves are measures of the concentration which must be applied outside the sheath or cuticle to produce the *true* threshold concentration at the nerve. Extrapolating to the normal oral taste receptors which control the behavioral thresholds, we may infer from the position of these behavioral thresholds, intermediate between those of sheath-protected and of desheathed nerves, that the nerves which govern them are not enclosed by normal sheath or cuticle but have more protection than bare nerve. This barrier may be to protect them from damage by high concentrations of chemicals, or it may be concerned with selection or differentiation between different types of taste stimulation.

In examining the curves lettered D and F, which represent the responses of desheathed nerve to the four chemicals tested, the most striking characteristic is the sharp drop in activity first seen after the threshold response. This rise to a peak of activity followed by a period of depression is repeated one or more times in varying degrees in all the curves before blocking occurs. A possible explanation is that the three or more peaks, particularly clear in the excised nerve cord preparations, may come from different groups of fibers each less sensitive or less exposed than the preceding. Each of these groups in turn could become active, reach a maximum, and then adapt or partially block to account for the peaks and depressions as the concentration of stimulating chemical is increased. In other words, parts of the nerve cord may be protected from chemical action by barriers comparable to the connective tissue sheath and the cuticle.

It is interesting to compare the behavioral thresholds with those obtained by Frings (1946) who also worked with cockroaches, using a different test method. In testing whether individual roaches would accept or reject test solutions offered them, Frings first determined the sucrose threshold by offering increasing concentrations of sucrose in water until an acceptable concentration was reached. This acceptance threshold was clear, but since individual roaches often refuse pure water, he had to use a different method to determine rejection thresholds. For this he

TABLE II

Behavioral thresholds determined by two methods

Thresholds	Sucrose	Sodium chloride			Hydrochloric acid		
	Acceptance	Acceptance	Rejection		Acceptance	Rejection	
			Partial	Complete		Partial	Complete
Reported here	.006	.004	.04	.6	.0006	.006	.4
Frings	.007			.2			.02

selected a sucrose concentration far enough above threshold to be always acceptable (0.1 M) and offered this with the addition of increasing concentrations of sodium chloride until it was rejected. This he took as the rejection threshold for sodium chloride. He repeated the experiments with hydrochloric acid and a large number of other salts and acids, and Table II shows a comparison of his results with the behavioral thresholds determined in these experiments.

It is at once apparent that there is very good agreement on the determination of sucrose against water, but the present method gives much more information about the sodium chloride and hydrochloric acid. As might be expected, Frings' lowest records for rejection lie within the range reported here as partial rejection. Frings was aware of the existence of the lower acceptance thresholds reported here but was unable to determine them because he could not get consistent feeding responses without the use of sucrose. It was the use of a large population of roaches instead of individuals which made it possible to get consistent feeding responses in these experiments without the use of sucrose.

It is quite possible that these thresholds as well as those of the nerve preparations may vary with different diets. In these experiments the same diet was supplied throughout, but it seems probable that any dietary change would affect both behavioral and nerve thresholds equally so that the same relationships would remain.

The methods used do not show whether the impulses from the nerve cord arise in the axons, dendrites or somata of the activated nerve cells. Since all impulses above the amplifier noise level were counted, it is not possible from the present data to estimate either the size of the responding elements or their relative number in the total population of active neurons. From other studies (Roeder, 1948) it seems probable that many compounds exert their effects on the dendritic or somatic regions of the central neurons.

These studies seem to indicate that the chemical thresholds for nerves unspecialized for gustatory reception are as low as, or lower than, those of the specialized receptors. On this basis we may conclude that taste or contact chemoreception depends on two factors. One of these, sensitivity, is also held by the neurons unspecialized for gustatory perception. Therefore, we can study it in nerve tissue other than the complex receptors which offer considerable technical difficulty. Furthermore, we can bring to bear on this study the vast amount of work which has previously been done on nerve tissue. The other quality, discrimination between different substances, remains a property of the receptor mechanism. However, it is hoped that its study has been simplified slightly by separating from it the factor of sensitivity which has often been considered an integral part of this mechanism.

SUMMARY

1. Application of increasing concentrations of sodium chloride to the normal intact tarsi of the American cockroach resulted in increased activity in the afferent fibers of the leg nerve when the threshold concentration was reached.

2. The threshold for this response was lowered by slitting the tarsal pads.

3. Although it may be presumed that there are no taste receptors on the nerve cord, when increasing concentrations of sodium chloride in saline solution were applied to an exposed section of intact nerve cord, it responded to a lower concentration than did the tarsal preparations.

4. The threshold of the nerve cord was further lowered by removing the connective tissue sheath which normally encloses it.

5. A section of the nerve cord, completely removed from the roach and exposed to the same concentrations of sodium chloride, responded at the same threshold concentration as the exposed nerve cord *in situ*, showing conclusively that the response did come from the nerve cord itself, not from adjacent chemoreceptors.

6. Behavioral experiments showed a response to the taste of sodium chloride at a threshold *higher* than that of the nerve cord preparations.

7. There was also an increase in the nerve activity from leg and nerve cord preparations in the same range of concentrations of sodium chloride which produced behavioral rejection.

8. Similar experiments with hydrochloric acid, sucrose and quinine, representing the sour, sweet and bitter sensations, also showed behavioral thresholds higher than those from the nerve cord preparations, and hydrochloric acid showed a correlation between nerve activity and behavioral rejection similar to that of sodium chloride.

9. It was concluded that high sensitivity to the four types of substances which produce the four taste sensations is inherent in nerves not normally connected with taste rather than being a special feature of the taste receptor, and that the basis for behavioral rejection may also be found in nerves not normally concerned with taste.

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HISTOPHYSIOLOGICAL STUDIES ON THE CORPUS ALLATUM OF LEUCOPHAEA MADERAE. I. NORMAL LIFE CYCLE IN MALE AND FEMALE ADULTS[1]

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In the course of previous work in our laboratory dealing with the functional and cytological properties of neuroendocrine systems, in particular the intercerebralis-cardiacum-allatum system of the insect, *Leucophaea maderae* (Scharrer, 1946b; Scharrer and Scharrer, 1944), it was observed that the corpora allata display an impressive variability in their morphology. The question arose whether differences in the size and histological appearance of these glands reflect differences in age, sex, functional state, etc., as is the case in an analogous organ, the anterior pituitary, or merely constitute the range of individual variation, or perhaps both.

The first indication that physiological state determines structure in the corpus allatum of *Leucophaea* was obtained by an analysis of the results of experiments in which nerves connecting this organ with the brain were severed. Following this operation, when performed at the appropriate time, the functional capacity of the gland was stepped up as demonstrated in last instar nymphs (Scharrer, 1946a), and there was a marked increase in glandular volume and relative cytoplasmic content (Scharrer, 1952). The conclusion seemed justified that, at least under the conditions of these experiments, a large gland in which the nuclei are loosely distributed represents a physiologically active gland.

This conclusion was borne out further by more recent studies (Engelmann, 1957) describing cyclic changes in the size of the corpora allata of normal adult females of the same species, *Leucophaea maderae,* in conjunction with reproductive processes.

Another investigation in which corpus allatum volume could be correlated with functional change concerns the effect of gonadectomy (von Harnack and Scharrer, 1956). These observations gave rise to two questions: (1) Are the increase in the size of the corpus allatum following gonadectomy and that occurring after nerve severance unrelated though comparable phenomena, or is there a mechanism involved which operates in both instances, resulting in a similar histophysiological response? (2) Is the volumetric increase observed in the corpora allata after both types of operation equivalent to that occurring under normal physiological conditions, *i.e.,* merely a sign of "activation," or does the response of the operated specimens go beyond the normal physiological range characteristic of the corpora allata?

In order to answer these questions data are needed, in addition to those reported by Engelmann (1957), concerning variations in the morphology of the corpora

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[1] Supported by research grants from the American Cancer Society and the U. S. Public Health Service.

[2] Grantee of the American Association of University Women.

allata in conjunction with physiological states throughout the adult life span of both males and females. Therefore, prior to reports on experimental results, the present paper deals with cytological evidence of secretory activity and variations in cell size, cell number, and nuclear-cytoplasmic ratio in the normal animal to the extent to which they can be examined with conventional techniques of light microscopy. These observations will serve as points of reference for subsequent papers which will deal with experimental observations.

Material and Methods

The material on which this investigation is based consists of 88 female and 46 male normal animals of known adult age. They were removed from stock colonies on the day of their emergence and isolated in pairs in pint-size jars. They were kept at room temperature on a routine diet of dog chow and fresh apple until the day of fixation. The animals were killed at selected intervals ranging from 0 to 599 days in the female, and from 0 to 471 days in the male series. In the majority of cases an autopsy was performed to determine the condition of the internal organs, particularly that of the reproductive organs of the females.

For the histological study of the corpora allata and associated endocrine organs, the heads were fixed in Zenker-formol. Among a variety of staining techniques tested the aldehyde fuchsin method (Gomori, 1950) proved most useful, when modified according to Halmi (1952) and Dawson (1953). The addition of Weigert's hematoxylin as a nuclear stain permitted the use of the sections for nuclear counts as well as a study of secretory products. Most of the tissues were cut serially at 7 μ, a small number of cases at 5 μ.

As a basis for comparison of the morphological characteristics of the corpora allata, three values were determined: the volume of both glands, the total number of nuclei, and their relative number per unit of tissue (nuclear-cytoplasmic ratio). In addition, nuclear size was measured in representative cases of large and small glands. Since it was considered desirable to include a large number of animals in the normal, as well as the experimental series which will be treated in subsequent papers, it became necessary to select methods of determining these quantitative values which afford a sufficient degree of accuracy without excessive expenditure of time.

(a) *Determination of volume*

The conventional method of estimating organ volume by measurements of each consecutive section in a series, when carried out in hundreds of specimens would be a staggering task. Therefore, methods based on the measurement of representative sections were explored for their validity in expressing organ volume. This seemed feasible because in our studies the aim was not so much to determine glandular volume as accurately as possible, but to select a convenient and reasonably valid numerical expression of quantitative differences. An additional advantage of such a simplified procedure is the possibility of using incomplete series of histological slides, a not infrequent result of the technical difficulties encountered with chitinous material.

The validity of the method to be adopted was tested as follows. To serve as a basis for comparison, the volumes of the corpora allata of 20 representative cases were calculated in μ^3 as accurately as possible by drawing each consecutive section with the aid of a camera lucida and measuring the drawings with a planimeter. By the inclusion of the extremes in this group of cases the range of variability was accurately established. Next, the same 20 cases were evaluated by measuring only intermittent sections. A comparison of results showed inaccuracies to be still rather small when only every ninth section was measured. The average number of sections per corpus allatum being 40 to 50, approximately 6 measurements were available for the calculation of the volume of each gland. Estimated values obtained by calculating the means between each of two consecutive measured sections were substituted for the values of the "skipped" sections. The inaccuracy of this procedure when compared with the results of measuring every section turned out to be small enough to permit the adoption of the abbreviated method for this and the following papers of this series.

(*b*) *Nuclear counts*

An accurate determination of the density of nuclear distribution meets with certain difficulties especially in small corpora allata poor in cytoplasm. In addition to the crowding of nuclei, a certain degree of irregularity in nuclear distribution has to be taken into consideration. Different methods of estimation were tested; the most reliable figures were obtained by direct counts under the microscope with the aid of a micrometer disc added to the eyepiece and of a tally counter. In each corpus allatum three representative sections were selected in approximately the same location, *i.e.,* the largest section in the middle of the series plus the fifth section from the middle section on either side. The total area of the three sections representing each corpus allatum and the sum of nuclei counted in this area permitted the calculation of the number of nuclei per mm.^{2}, a figure which represents a fairly good index of nuclear density. These values are recorded in Figures 1 and 2.

In order to determine whether a volumetric increase in the corpora allata is due solely to a rise in cytoplasmic content or whether this is accompanied by an increase in nuclear number, an at least rough calculation of the absolute number of nuclei present in each pair of corpora allata studied became desirable. For this the following formula suggested by Engelmann (1957) was used:

$$N = \frac{N' \times V}{A \times (T + 2r)},$$

where N = total number of nuclei per pair of corpora allata, N' = number of nuclei counted in A (total area of three sections selected for nuclear counts), V = volume of both corpora allata, T = thickness of sections, $2\ r$ = average nuclear diameter.

RESULTS

a. Females

The aim of this study was to analyze in detail the periodic changes in the corpora allata in the course of one reproductive cycle, and to determine whether essentially the same pattern obtains in successive cycles throughout the adult life span. It soon became apparent (and was subsequently substantiated in our

TABLE I

Quantitative changes in the corpora allata of 26 females of Leucophaea maderae of varying adult age (maximum 599 days) grouped according to different stages in the reproductive cycle

	Number of animals	Range in volume of corpora allata (in mill. μ^3)	Range in number of nuclei	Range in nuclear-cytoplasmic ratio (nuclei/mm.^{2})
Group A (small eggs in ovary; beginning of cycle)	8	5.0–10.8	3657–6411	733–2034
Group B (growing eggs in ovary)	3	9.0–12.4	4137–9488	864–1558
Group C (pre-ovulatory stage)	3	15.3–37.0	7017–15,591	825–987
Group D (pregnancy)	12	3.9–17.3	3124–11,361	1155–1978

experimental work) that the corpus allatum of *Leucophaea* shows a fair degree of structural variability within groups of specimens selected under comparable physiological conditions. This fact had to be taken into account in the search for significant correlations with functional states and necessitated the use of larger samples than would otherwise be called for.

There is also a certain degree of individual variation in the timing of the alternating periods of ovarian activity and quiescence characteristic of the reproductive activity of *Leucophaea*. Therefore, an analysis of the first cycle which begins after the emergence of the adult offers advantages because of the greater ease of dating. For the study of subsequent cycles the "adult age," *i.e.*, the time elapsed since emergence, is less significant than the conditions of the ovary at the time of fixation. Consequently the selection of appropriate stages in these later cycles is facilitated by the recording of preceding parturitions. This is taken into account in the arrangement of the data summarized in Figure 1. These include 62 females killed at intervals of a few days during the first and second reproductive cycles. The cases illustrating the first cycle are arranged according to adult age (days elapsed between emergence and fixation of the animal), those of the second cycle are grouped in reference to the interval between preceding (*i.e.*, first) parturition and fixation.

An additional 26 animals studied in this series encompass the remainder of the entire adult life span. The oldest specimens represent extremes in longevity obtained from a large collection of dated females and are of particular value in the search for possible changes in corpus allatum function with increasing age. This group of older females, dated according to adult age, does not lend itself for the same graphic representation as the younger specimens, since the number of reproductive cycles they had completed was not recorded in all cases. This group is, therefore, not included in Figure 1. The values obtained for these animals grouped according to phases of the reproductive cycle are summarized in Table I.

As can be seen from Figure 1, within 30 days after emergence the volume of the corpora allata rose from an average value of 4.2 million μ^3 (minimum 3.5) in the very young female to an average value of 15.3 million μ^3, (maximum 16.6) corresponding to the time when the largest oocytes had almost reached their maximal size.

After ovulation, the corpora allata returned to approximately the same size

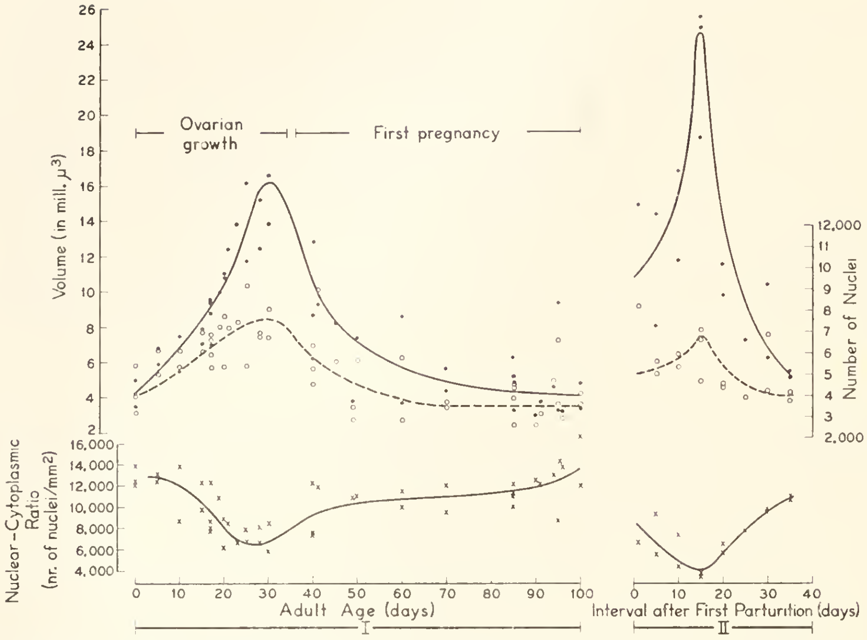

FIGURE 1. Graphic representation of morphological changes in the corpora allata of adult females of *Leucophaea maderae* in conjunction with reproductive cycles. ——●——●—— = volume of both corpora allata in million μ^3; -----○-----○----- = number of nuclei, calculated for both glands; ——×——×—— = nuclear-cytoplasmic ratio (number of nuclei/mm.^{2}). Diagram includes (I) entire period of first reproductive cycle (from emergence of adult to first parturition at about 100 days of adult age), (II) second reproductive cycle (from first parturition to early part of second pregnancy). In this and subsequent figures of this series the curves do not represent mathematically correct summaries of the quantitative data which do not lend themselves to such treatment; they are merely intended to facilitate the visualization of the changes over the periods indicated. Note that volumetric changes are paralleled by changes in nuclear numbers and in nuclear-cytoplasmic ratios. For details see text.

as those of newly emerged females. This level was maintained throughout "pregnancy" to be followed by a more pronounced and more rapid rise (maximum volume 25.5 million μ^3) reached after 15 days in the pre-ovulation period of the second cycle. A study of this and older groups (Table I) indicated that essentially the same periodic changes in corpus allatum volume in conjunction with alternating phases of activity and inactivity occur in consecutive reproductive cycles. The first cycle differs from the subsequent ones only in degree in that the volumetric maximum is lower and is reached more slowly. In this respect our data agree with those of Engelmann (1957).

We came to different results, however, with respect to the remarkable cellular changes accompanying the periodic increase and decrease in corpus allatum volume. While it is true that the nuclei are more widely spaced in large, active corpora allata than in small, the rise in glandular volume is not exclusively due to an increase in the amount of cytoplasm. The contribution made by the nuclei in this growth process is primarily by a rise in their number, and less by an increase in their size. In our series, the total number of nuclei estimated per pair of corpora allata rose from a minimum of 3128 in newly emerged females to a maximum of 9220 in the first, and of 8236 in the second pre-ovulation period.

The highest nuclear count of the whole series (15,591) belongs to a female which also has the highest volumetric value for normal corpora allata (37 million μ^3). This case is noteworthy in that it is a female with the exceptional adult age of 599 days carrying large ova at the time of fixation. Its corpora allata, in addition to the large number of normal sized nuclei, contain a giant nucleus (diameter 46 μ) comparable to those described by DeLerma (1932) and Palm (1947) in *Gryllotalpa*. The smallest number of nuclei (2782) occurred in small corpora allata of a 90-day-old female fixed before the onset of the second cycle. This shows the interesting fact that, with decreasing organ volume in pregnant females, the number of nuclei falls to reach again the level characteristic of very young adult specimens.

To sum up, during subsequent periods of activation and quiescence of the corpora allata the increase and decrease in organ volume is accompanied by periodic changes in nuclear numbers. The fact that the volumetric range (3.0–37.0 million μ^3 for both corpora allata) surpasses that of the nuclear numbers (2782–15,591) expresses itself in marked differences in nuclear distribution: Generally speaking, the larger the glands, the fewer the nuclei counted per mm.^{2} (range: 3698–16,765), and the higher the absolute and relative cytoplasmic content of the corpus allatum tissue. The cyclic fluctuations in nuclear numbers are so pronounced that cytological manifestations of these changes should be expected. Signs of mitotic activity, to account for the cell multiplication calculated, have been observed both in our normal and colchicine-treated adult specimens. The number of mitotic figures counted in the corpora allata is altogether small; while they appear to be more frequent during organ growth, mitoses are not entirely restricted to this period.

Conversely, signs of nuclear destruction (pycnosis) seem to be more pronounced in corpora allata returning to the inactive state, but are also occasionally found in growing or maximally active glands. This means that growth and regression of the corpora allata are not solely responsible for the fluctuations in nuclear numbers. The shifts in the frequency of mitotic and pycnotic nuclei can perhaps be better understood in conjunction with the cytological manifestations of the secretory activity of these glands.

While no comprehensive analysis of the elaboration of the secretory product by the corpora allata of *Leucophaea* is intended in this paper, certain statements may be made. Methods such as Gomori's chrome hematoxylin phloxine, Foot's modification of Masson's trichrome, or hematoxylin and eosin stain are not suitable for the demonstration of secretory materials in these glands. With the aldehyde fuchsin technique, distinct small granules can be demonstrated within the cytoplasm which stain from a rather deep purple to lavender. On occasion larger green staining droplets are observed. Newly emerged animals in our material do not show these granules; in older specimens their number and distribution appear to depend on the functional state.

During what seems to be a rather short and early phase in the secretory cycle certain corpus allatum cells stand out because their cytoplasm is densely packed with stainable granules. Their presence permits the tracing of cellular processes which are the longer the more central the location of the cell within the gland. In other words, the corpus allatum cells in their mature form are stellate, and obviously release their secretory products by means of processes which end perpendicularly to the surface of the organ. This stellate cell shape cannot be readily ascertained in the absence of secretory granules, since cell boundaries are not always easily observed in the corpora allata. Many specimens show a more widespread distribution of granules which in sections can no longer be brought in spatial relationship with specific gland cells.

In cells presumably representing later stages in the secretory cycle the cytoplasm, instead of being homogeneous, assumes a more or less "stringy" appearance interspersed with vacuoles. These strands of cytoplasm form a characteristic pattern as though applied by strokes of a brush in a direction from the center to the periphery of the gland. It is along these "lines of flow" (Mendes, 1948; Özbas, 1957) that the secretory granules are now oriented, and their direction corresponds to that of the cell processes mentioned above. The fact that the granules tend to become lined up in greater numbers in the periphery of the corpora allata also speaks for their eventual release into the surrounding body fluid.

A further point of interest is that the nuclei of cells containing many secretory granules often appear pycnotic. These and additional pycnotic nuclei, not surrounded by secretory granules, range from slightly shrunken structures to homogeneous intensely staining bodies. Thus, there appears to be in adult corpora allata a continuous cellular turnover, whereby cells becoming exhausted in the process of their secretory function are replaced by the mitotic activity of younger probably non-secreting cell elements. The rate of cellular turnover seems to fluctuate in the course of a reproductive cycle, with the result that activation of the corpora allata is accompanied by increase, and return to inactivity by a decrease in cell numbers.

A further question concerns possible fluctuations in nuclear diameters in relation to periodic changes in organ volume. Measurements of representative cases have shown the nuclear diameter to vary only moderately in normal specimens (from 6.4 μ to 8.2 μ; mean 7.0 μ). The nuclei of any given specimen may fall within this range, and no definite relationship between organ volume and average nuclear size could be established. The measurements given do not include those of rarely found giant nuclei which seem to occur characteristically in old specimens (see above).

Of all the periodic changes in the appearance of the corpus allatum of *Leucophaea* the one most readily observed is that of the nuclear-cytoplasmic ratio. Thus, even without quantitative determinations, the large, *i.e.,* active corpus allatum can be easily distinguished from the inactive on the basis of its histological appearance.

b. Males

The 46 male specimens studied range from an adult age of 0 to 471 days (Fig. 2). As in the female series, the corpora allata of animals of the same age may show a certain variability in size and nuclear distribution. The smaller average body size of males is reflected in lower corpus allatum values. Shortly after emergence the glands begin to grow, but this period is shorter than in females. After about 10 days a peak is reached which, on the average, amounts to a volumetric increase of 2½ times over the initial volume. After this, the values level off to fluctuate only mildly throughout the adult stage. The volume of the largest pair of glands measured in this series (9.0 million μ^3) is about four times that of the smallest (2.1 million μ^3). As one might expect, in analogy to the situation in females, the variability in total nuclear counts is somewhat lower in degree than that in volume, but it is nevertheless considerable (range: 2032 to 7000).

Thus, if arranged in the order of increasing volume, male corpora allata also show a gradual increase in the relative amount of cytoplasm. The number of nuclei per mm.^{2} ranges from 7696 to 17,131. Inasmuch as male corpora allata are on the average smaller than female, their nuclei are generally more crowded. The highest density occurred in a case (adult age: 51 days) which also had the largest absolute number of nuclei; the corpora allata were of medium size. The loosest arrangement of nuclei was observed in an old specimen (adult age: 396 days) with large corpora allata (7.4 million μ^3). The relative cytoplasmic content was almost as high in a male (adult age: 10 days) which had the largest corpora allata in the entire series.

Figure 2 shows quite clearly that the rise in corpus allatum volume during the first ten days of adult life is accompanied by an increase in nuclear numbers and a decrease in the number of nuclei per unit of tissue. The reverse trend occurs after the peak, *i.e.,* in males older than 10 days, but as a group these males do not entirely return to the situation characteristic of newly emerged animals. In principle, the male corpora allata show the same relationships between organ size, number of nuclei, and density of nuclear arrangement as the females, but this relationship is not so pronounced. It is interesting, for example, that within a medium size range (4–5 million μ^3) which encompasses the majority of males

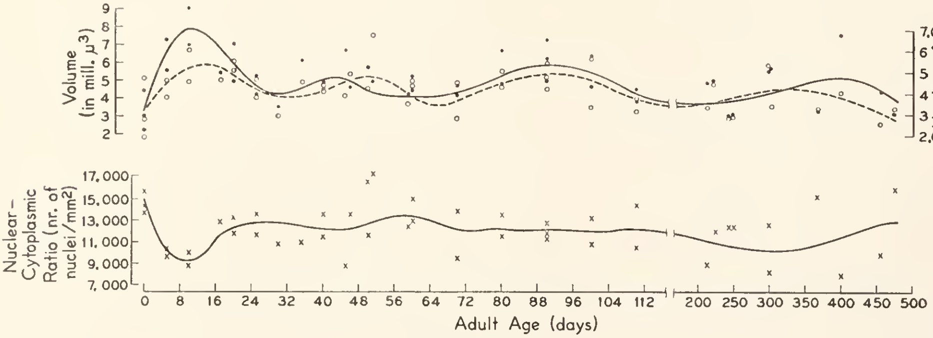

FIGURE 2. Diagram showing morphological characteristics of corpora allata of males of *Leucophaea maderae* ranging in adult age from 0 to 471 days. After a small initial rise (maximum at 10 days) the volumes (——●——●——) level off to fluctuate around a mean value maintained throughout the adult life span. A lack of distinct cyclic activity is also apparent from the values expressing nuclear numbers (-----○-----○-----) and nuclear-cytoplasmic ratios (——×——×——).

studied, the nuclear-cytoplasmic ratio shows the highest degree of variability as compared to that in groups with smaller or larger glands.

The data mentioned so far do not provide evidence for the existence of cyclic activity changes in the corpora allata of adult males. This also applies to the cytology of secretory processes. Purple-staining granules in the cytoplasm have been found in varying amounts in animals of different ages with the exception of newly emerged specimens. While indicating the existence of a secretory function in male adult corpora allata, these cytological phenomena are less impressive than those in females which they resemble in principle. The fact that signs of secretory cycles as well as volumetric and nuclear changes are less apparent in males is related to the greater range in the response of female corpora allata to varying physiological conditions.

Discussion

In most species of insects studied, extirpation and implantation experiments have established the control of reproductive processes by a hormone of the corpora allata (for review see Scharrer, 1955). There are also numerous indications that, at the height of their "gonadotropic" activity, the corpora allata of females are larger than when they are inactive. This was reported among others by Ito (1918), Wigglesworth (1936), Thomsen (1942), Palm (1947), Mendes (1948) and Kaiser (1949). However, most of these and more recent studies (Müssbichler, 1952; Nayar, 1956; DeWilde, 1954; Brandenburg, 1956; Lukoschus, 1956; Lhoste, 1957) do not extend beyond a relatively short interval between emergence and oviposition. A more detailed analysis of this relationship was carried out by Engelmann (1957) who correlated corpus allatum volume and structure with ovarian activity in *Leucophaea maderae,* the species which was also used in the present study. During the long life span of this "ovo-viviparous" species, periods of ovarian quiescence during "pregnancy" alternate with those of activity in which the terminal oocytes grow and deposit yolk, and the accessory sex glands produce secretory material. Only this active phase in the reproductive cycle requires the presence of the corpus allatum hormone while the maturation of the embryos proceeds without it (Scharrer, 1946b). When Engelmann found large corpora allata with relatively high cytoplasmic content in females approaching ovulation, and small glands with densely packed nuclei during pregnancy, he concluded that "activation" of the corpora allata is characterized by an increase in the amount of cytoplasm while the number of nuclei remains constant. The present study, based on a larger material, confirms these earlier results as far as the periodic changes in corpus allatum volume are concerned; it also offers evidence that these changes continue beyond the period (135 days after emergence) analyzed in Engelmann's study, *i.e.,* throughout the adult (reproductive) phase of the insect. In addition, our results demonstrate a participation of the nuclei in the activity cycles of the corpora allata. The role of the nuclei could be ascertained only by quantitative methods in a sufficiently large material, since cell divisions are not observed frequently enough in normal adult specimens. Nuclear counts have shown that, in the first as well as subsequent reproductive cycles, an up to four-fold increase in the number of nuclei may take place during the phase of growth and activation of the corpora allata. The decrease in organ volume which follows is accompanied by a corresponding decrease in nuclear number. These differences in total nuclear counts are so large that they allow a considerable margin of error which cannot be avoided in the calculation of these figures.

While our cytological observations do not lend themselves to a quantitative evaluation of nuclear changes during the activity cycles of the corpora allata, it is significant that signs of mitotic activity are more prominent in growing glands and pycnotic nuclei are more conspicuous in corpora allata returning to the inactive state. These observations in correlation with the study of the cytology of the secretory cycle lead to the conclusion that corpus allatum cells are used up and replaced during the adult life of the animal. Cyclic changes in the rate of this cellular turnover which accompany secretory cycles in these glands account for the periodic increase and decrease in nuclear numbers and accompanying fluctuations in organ volume.

By comparison, nuclear size showed less variability in our material, so that the main contribution of the nuclei toward increase in organ volume is due to a rise in their number. Taken as a whole, the participation of the nuclear component stays behind that of the cytoplasm which shows not only pronounced quantitative changes but undergoes qualitative transformations during the process of elaboration of secretory granules.

The use of the aldehyde fuchsin technique in the present study for the first time permitted the demonstration of secretion granules in the corpora allata of *Leucophaea*. Although details of the whole secretory cycle still need to be worked out, present evidence supports the view that the active principle is elaborated in the cytoplasm of stellate cells and released into the body fluid surrounding the surface of the gland. Depending on the more peripheral or central location of a cell, its processes may be short or long. They account for the characteristic structural pattern of the gland during certain stages of the cycle in which strands of cytoplasm directed toward the periphery and interspersed with vacuoles are delineated by rows of secretory granules which become more numerous in the periphery of the gland. It is uncertain whether a gland cell remains active only during one secretory cycle or not, but the frequent observations of more or less pycnotic nuclei in cells filled with the secretory product indicate that the cells may become exhausted and are replaced by cells resulting from mitotic divisions in the adult gland. The observation of cytoplasm becoming vacuolized during phases of activity as well as the arrangement of secretory granules along "lines of flow" (determined by the peripheral direction of the cell processes) is in agreement with the findings of other authors in different species of insects (Mendes, 1948; Özbas, 1957).

Thus, in the cyclic activity of the corpora allata of adult females of *Leucophaea* a number of factors are involved. The question arises which of the changes observed express the physiological activation of the corpus allatum. This question cannot be fully answered as yet, but certain known data are of interest in this connection. Increase in nuclear number without relative cytoplasmic increase can be achieved in adult female glands by the implantation of prothoracic glands (Engelmann, personal communication). The absence of ovarian stimulation in animals thus treated indicates that in the corpus allatum of adult *Leucophaea* the relative cytoplasmic increase is an important prerequisite for its activation. The possibility that it is the only one is illustrated by the situation in those species where corpus allatum growth in adults is said to take place solely by an increase in cell volume and nuclear volume, but not in cell number (Brandenburg, 1956; Lukoschus, 1956). It is evident from Engelmann's data as well as our own (Fig. 1) that at the first peak of activity the corpus allatum volume does not reach the same level as at the second and subsequent ones. Conversely, more time is required for the stimulation of the ovary during the first reproductive cycle than later on. It seems that the corpus allatum may require a short period after emergence to complete its adult development, as has been postulated also for other organs (Rockstein, 1956). The gland may, therefore, not be capable of complete "activation" until after the first cycle.

The possibility that such post-emergence maturation takes place is also indicated by the moderate rise in volume and nuclear numbers occurring in male corpora allata within the first ten days of adult life. Our own observations in this respect are in line with those of Engelmann (personal communication) in *Leucophaea* and by Mendes (1948) in *Melanoplus*. At any rate, no interpretation other than one of tissue maturation can be given for the initial volumetric increase of the corpora allata of adult males, as long as their functional role is so little understood. All we know is that the male reproductive activity in *Leucophaea* is undisturbed after allatectomy (Scharrer, 1946b), and that certain data suggest a relationship between corpora allata and metabolic processes (Samuels, 1956). A sustained control of metabolic functions by the corpora allata might well account for the picture of "mild activity" frequently observed in histological preparations of adult male glands of every age. This steady appearance, which is in contrast to the cyclic pattern in the corpora allata of adult females, concerns range in organ volume and nuclear number as well as cytological manifestations of secretory activity.

In connection with the observation in both sexes of corpus allatum stimulation following emergence it is of interest that this early adult period in *Leucophaea* also differs from later ones with respect to certain metabolic data, such as the animal's lipid content (Scharrer and Wilson, unpublished data).

The present study illustrates that the histophysiological approach whose value is well recognized in vertebrate endocrinology is equally fruitful in the exploration of endocrine mechanisms in insects. In the special case of the corpora allata of *Leucophaea,* known variations of their activity are paralleled by marked changes in the volume of the entire organ, the number of cells, the nuclear-cytoplasmic ratio, and the cytology of the secretory process. The subsequent papers will be concerned with corpus allatum structure following experimentally induced changes in the normal pattern of activity and quiescence of this gland.

Summary

1. The corpora allata of *Leucophaea maderae* display a remarkable degree of structural variability in conjunction with changing functional states. This is particularly apparent in adult females, where a regular sequence of activity and inactivity of these glands parallels alternating phases of ovarian development and quiescence.

2. The volume of active corpora allata surpasses that of inactive glands beyond the range of individual variation, which is considerable. The volumetric rise signalling activation is accomplished to a large extent by an absolute and relative

increase in cytoplasmic content which results in a characteristic "loose" distribution of the nuclei.

3. The present study shows further that the nuclei participate significantly in the cyclic changes of these organs. During each growth period, the nuclear numbers increase up to several times the original values. When, after ovulation, the corpus allatum returns to a state of inactivity which is maintained during pregnancy, the nuclear-cytoplasmic ratio returns to a level characteristic of the newly emerged female. The accompanying reduction in cell number to the initial level is evident not only from a drop in nuclear counts but from the observation of pycnotic nuclei.

4. The differences in the frequencies with which nuclear pycnosis on one hand, and mitotic figures on the other are observed in various stages suggest the existence of a cellular turnover which seems continuous but whose rate changes periodically. During activation of the corpus allatum, when secretory products are elaborated, the increase in cell number surpasses the rate of cell destruction. In the regressing gland the latter process predominates over that of cell replacement.

5. With the use of a modified aldehyde fuchsin technique distinct secretory granules have been demonstrated in the corpus allatum cells of *Leucophaea*. The granules line up along processes of the cells which are directed to the surface of the corpus allatum. This fact, as well as the accumulation of stainable granules in the periphery of the gland, speaks for the release of the active substance into the surrounding hemolymph. The occurrence of pycnotic nuclei in cells filled with secretion granules suggests that these gland cells may become exhausted fairly quickly, perhaps in the course of one secretory cycle.

6. By comparison with the situation in the females, the corpora allata of adult males show considerably less variability. Soon after emergence, a short period of "activation" seems to occur, as judged by the same structural characteristics as in the females. After that a fairly constant level of presumably mild activity appears to be maintained throughout adult life. Since the available information on the functional role of the corpora allata in male adult animals suggests no pattern of periodicity, the lack of distinct cyclic changes in the morphology of these glands is not surprising.

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HISTOPHYSIOLOGICAL STUDIES ON THE CORPUS ALLATUM OF LEUCOPHAEA MADERAE. II. THE EFFECT OF STARVATION[1]

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The corpora allata of *Leucophaea maderae* undergo marked histological changes in conjunction with various phases of the reproductive cycle (Engelmann, 1957; Scharrer and von Harnack, 1958). Activation of the corpus allatum is characterized by an increase in organ volume, in nuclear number, and in absolute and relative cytoplasmic content. These changes, as well as the cytological manifestations of secretory activity, reach a peak shortly before ovulation. During the subsequent period of pregnancy, when the ovaries presumably receive no hormonal stimulation, the corpora allata return to the inactive condition (small size, dense nuclear arrangement) in which they remain until the onset of the next reproductive cycle.

The decision as to whether or not the corpora allata become activated at any given time resides in the central nervous system which, under certain conditions, exerts a restraining influence on these glands. In addition, nervous or neurohumoral stimuli appear to be necessary to sustain the activity of the corpora allata (Scharrer, 1952; Engelmann, 1957). The type of message sent to the corpora allata is determined by a variety of afferent impulses from the external and internal milieu (see Scharrer, 1958, 1959). One of these is the nutritional state of the animal.

During a period of total starvation egg development is suppressed in *Leucophaea* (Scharrer, 1946); however, the ovary remains capable of responding to implanted active corpora allata (Johansson, 1955). From this result one can conclude that the absence of nutrients acts as a stimulus to the brain eliciting an inhibitory message to the corpora allata.

It was of interest, therefore, to examine the effects of inanition on the morphology of the corpora allata of adult females of *Leucophaea maderae* (von Harnack, 1958). The present study is concerned with two aspects of this problem: (1) Does prolonged total starvation affect the structure of the corpora allata, either to or perhaps beyond the point of preventing their activation? (2) How do corpora allata, kept inactive by starvation for a considerable period, respond to the resumption of a normal diet?

Material and Methods

The material on which this investigation is based consists of two series (A and B), one in which the animals were subjected to total starvation for various periods

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[1] Supported by a Research Grant from the U. S. Public Health Service administered by Dr. Berta Scharrer.

[2] Grantee of the American Association of University Women.

of time, and another in which a period of starvation was followed by return to a normal diet. All animals used were adult females removed from the stock colonies on the day of emergence and isolated in pint-size jars. At the same time the period of starvation was initiated during which the majority of animals received only water; a small number was deprived of water as well as food.

Series A was used for the study of the influence of prolonged total starvation on the morphology and function of the corpora allata. It consisted of 52 females on a water diet, and of 31 females starved and dehydrated at the same time. The animals were fixed at intervals ranging from 5 to 95 days following the beginning of the starvation period, *i.e.,* the specimens surviving longest had reached an adult age at which normally the first reproductive cycle would have been completed. Animals showing increasing signs of weakness before being fixed could be presumed to have largely exhausted their nutritional resources. As might be expected, the point of exhaustion was reached sooner in the dehydrated group.

Series B gives information on the response of the corpora allata to the resumption of a normal food intake following an extended starvation period. In this group 51 newly emerged females received nothing but water for 30 days. They were then returned to a regular diet of dog chow and apple; they were kept together with normal males, and allowed to survive for up to an additional 90 days. In both experimental series, fixation was scheduled at five-day intervals, except during periods when more pronounced structural changes of the corpora allata were observed and, therefore, one- or two-day intervals became desirable.

Autopsies were performed in all cases in order to ascertain the condition of the reproductive organs. The histological procedure, and the method of quantitative evaluation of the corpora allata were the same as reported in the preceding paper of this series (Scharrer and von Harnack, 1958).

RESULTS

Series A: Starved animals

In contrast to the corpora allata of normally fed females which reach a four-fold increase in volume within 30 days after emergence, the corpora allata of starved females are noticeably suppressed (Fig. 1 and Fig. 3). The situation in the group of 52 animals kept on a water diet was as follows. During an initial period of 15 days, when inanition had not yet become effective, the corpora allata grew at a rate comparable to that in normally fed animals, *i.e.,* their volume doubled. After that the corpus allatum volume of starved females showed a gradual but continuous decrease. At 30 days of adult age the corpora allata had returned to the level of the newly emerged female, and the longest survivor, fixed after 95 days, had reached a minimal corpus allatum volume of 1.7 million μ^3. This means that prolonged starvation had caused a decrease of corpus allatum volume to about one-half of the minimal size found in normally fed adult females.

The initial rise in corpus allatum volume of the starvation series is paralleled by an increase in nuclear numbers which compares with that in the normal control series. The turning point at which the nuclei begin to decrease in number occurs sooner in the experimental group (Fig. 1) than in the controls. Subsequently the nuclear counts return to a value characteristic of the normal inactive gland, while the cytoplasmic content drops considerably below the normal baseline. The

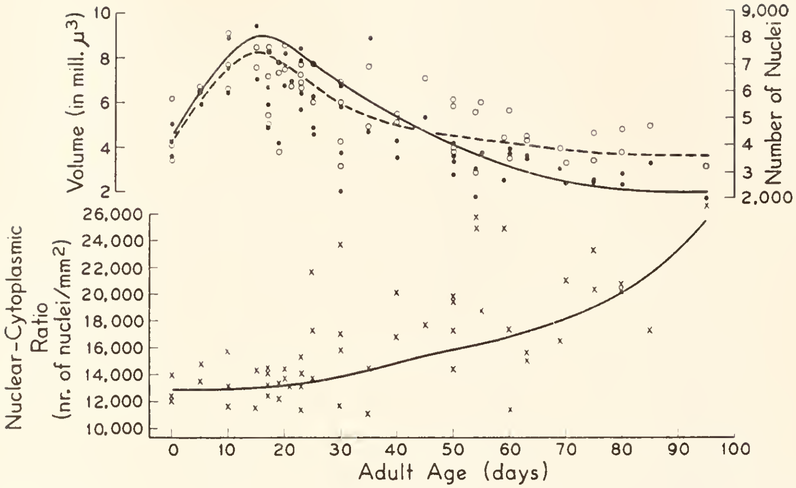

FIGURE 1. Diagram indicating morphological changes in the corpora allata of adult females of *Leucophaea maderae* which received only water from the day of emergence. Note that the volume of the corpora allata (——●——●——) and their number of nuclei (-----○-----○-----) fall after a moderate initial rise. ——×——×—— = nuclear-cytoplasmic ratio. For comparison with normal controls see Figure 3.

result is a very crowded nuclear arrangement in the corpora allata of specimens fixed after excessively long periods of starvation.

These stepwise histological changes are enhanced in the starved-dehydated group. When combined with dehydration, the first manifestations of inanition become evident earlier; the turning point in corpus allatum volume occurs already at about 12 days of adult age and appears accordingly somewhat lower on the curve (Fig. 3) than that in the starved-hydrated group. Thus the decline in corpus allatum volume which follows the moderate peak in the dehydrated group runs roughly parallel to that in the hydrated group and reaches the endpoint sooner (Fig. 3). Another indication of the aggravating effect of dehydration on starving animals was the extreme crowding of nuclei which made quantitative estimates of reasonable accuracy impossible. Nuclear counts were, therefore, omitted in the starved-dehydrated group of animals. In the present as well as earlier experimental series survival rates were consistently lower in starved-dehydrated than in starved-hydrated groups. The longest survival recorded in the dehydrated group was only 65 days.

As was to be expected from preceding studies (Scharrer, 1946; Johansson, 1955), none of the females deprived of food from the day of their emergence showed signs of reproductive activity. On autopsy, only small undeveloped eggs were observed in the ovaries, and the accessory sex glands contained no appreciable amount of secretory material.

Series B: Starved and re-fed animals

In this group of females the return to a normal diet occurred after a starvation period of 30 days in which only water was available; *i.e.,* at a time when the

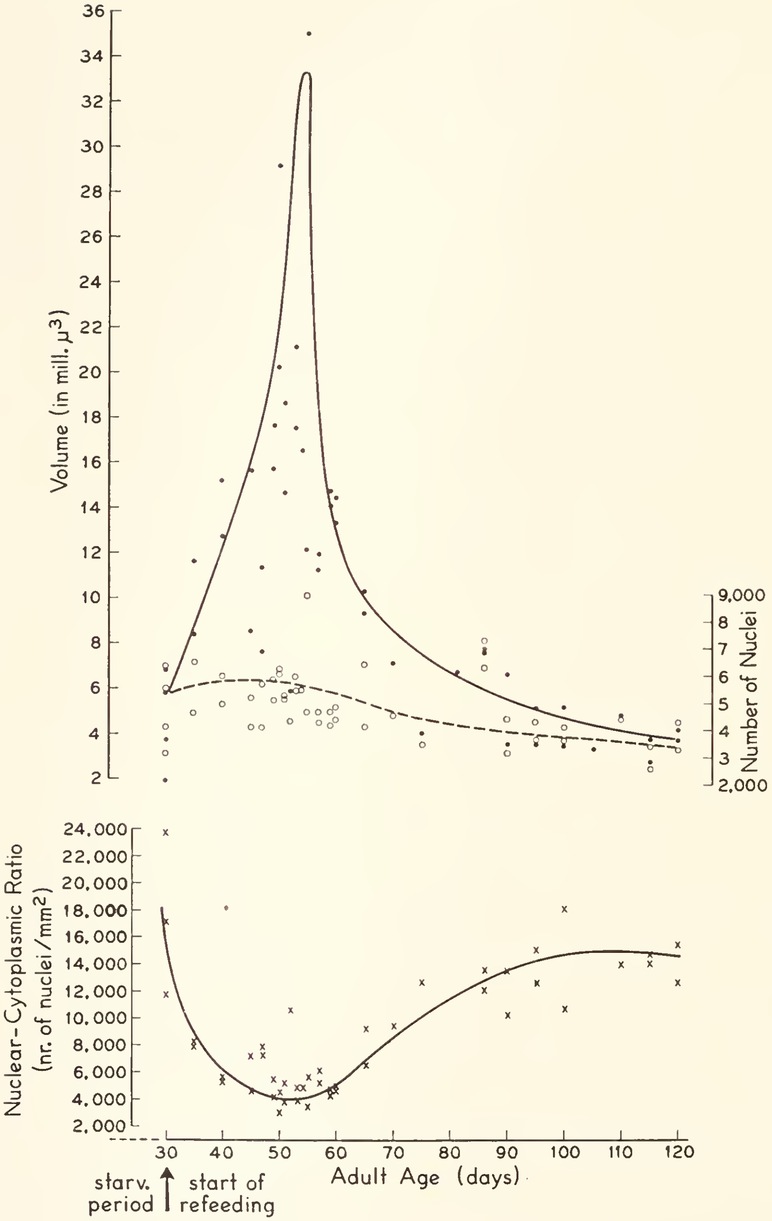

FIGURE 2. Diagram showing the response of the corpora allata in adult females of *Leucophaea maderae* which were starved, with access to water, for 30 days following emergence and were then returned to a normal diet. ——●——●—— = volume of both corpora allata; -----○-----○----- = number of nuclei of both corpora allata; ——×——×—— = nuclear-cytoplasmic ratio (number of nuclei/mm^2). Compare volumetric changes with those of normal controls (Fig. 4).

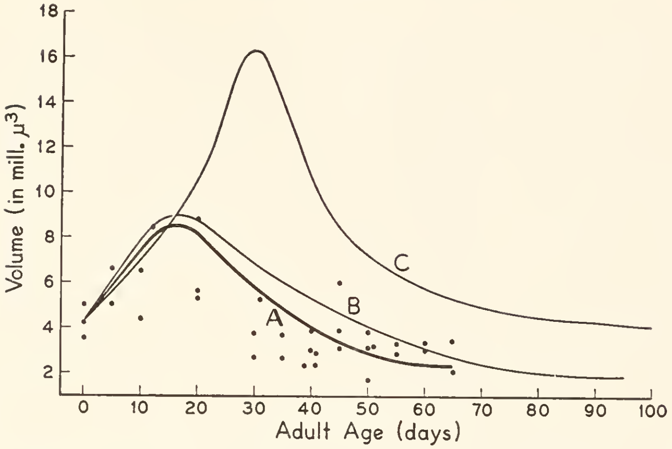

FIGURE 3. Diagram illustrating changes in the volume of both corpora allata of females of *Leucophaea maderae* which were starved and dehydrated following emergence (curve A). Compare with group of starved-hydrated animals (curve B) and with normally fed control group (curve C).

corpus allatum volume as well as the nuclear counts had almost returned to baseline levels characteristic of the normal inactive gland. Re-feeding resulted in a rather dramatic response of the corpora allata. Within 15–20 days, a peak was reached which represented a seven-fold increase in volume (Fig. 2). This considerably surpasses the peak characteristic of the growth curve of the first reproductive cycle under normal feeding conditions (four-fold increase in volume). It compares favorably with that of the second normal cycle which it also resembles with respect to the steepness of the slopes (Fig. 4). It appears as though the starved animal, on resumption of feeding, is capable of "making up for lost time" and does not have to start its reproductive period as "gradually" as the normal animal.

The rapid rise of corpus allatum volume in the starved and re-fed series is not accompanied by a correspondingly high increase in nuclear number (Fig. 2). Consequently, the largest corpora allata have an exceptionally high content in cytoplasm, and accordingly the nuclei are more widely spaced than in the most active glands of the first reproductive cycle of the normal series.

The full-scale activation of the corpora allata in this first reproductive cycle of the starved-re-fed animals promptly led to stimulation of the ovaries; ovulation occurred within about 25 days after the resumption of feeding.

DISCUSSION

In the present as well as earlier experiments (Scharrer, 1946; Johansson, 1955; Willis and Lewis, 1957), the roach *Leucophaea maderae,* when subjected to total starvation, showed a considerable capacity to survive. If water was provided, adult females which were starved from the day they emerged lived for up to three months at room temperature. This period was shortened by about three weeks, when the insects were deprived of water as well as solid food. In *Leucophaea maderae,* as in certain other insect species, eggs do not develop in the total

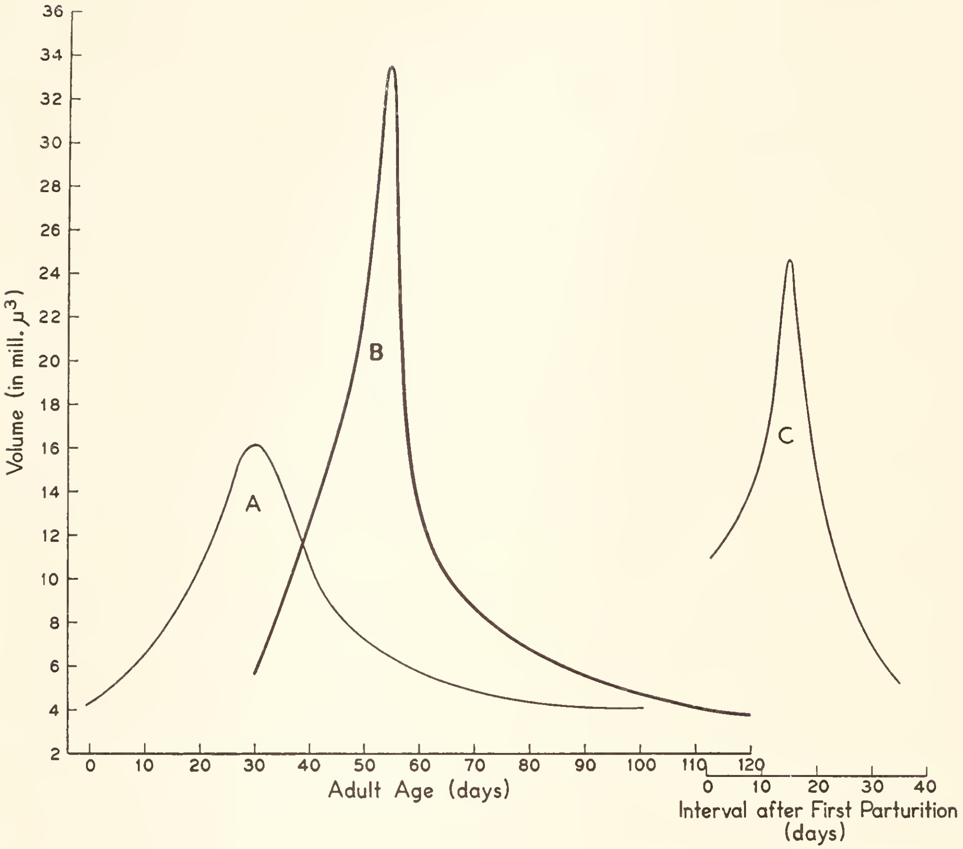

FIGURE 4. Diagram showing the pronounced rise in corpus allatum volume of starved-re-fed females (curve B), in comparison with corpus allatum growth in first normal (curve A) and second normal reproductive cycle (curve C).

absence of nutrients (Scharrer, 1946), unless active corpora allata are implanted (Johansson, 1954; 1955), or the starving insect's own corpora allata are released by surgery from the action of the restraining nerves (Johansson, personal communication). These results demonstrate that (a) the gonads of starved animals have the capacity to respond to gonadotropic stimulation, and (b) the initial moderate rise in corpus allatum volume observed in starving *Leucophaea* females seems to represent a degree of activation insufficient to elicit ovarian response.

The longer the period of inanition, the smaller and the poorer in cytoplasmic content are the corpora allata. It is difficult to estimate the number of nuclei present in these small organs, and to demonstrate possible signs of secretory activity. At the endpoint, beyond which survival was no longer possible, the corpora allata had only about one-half the volume of normal female "inactive" glands. It cannot be determined with certainty whether or not this decrease below the normal range of corpus allatum size is nothing more than a general effect of inanition shared by other organs of the body. An attempt was made to measure organs in the vicinity of the corpora allata, such as the musculature of the head. A comparison of the diameters of muscle fibers in normal and starved specimens suggests that some "wasting" occurs in the latter. This is also evident from the larger spaces between muscle fibers in drastically starved animals. However, since the individual muscle elements vary in width, a reasonably accurate estimation of the degree of shrinkage is not possible.

What was said so far applies to starved animals receiving water, and to an even larger degree to starved-dehydrated specimens. Their corpora allata do not even reach the size of the starved-hydrated group, and the decline in volume (and activity) occurs proportionately sooner. A comparable dependency of the morphology and function of the corpus allatum on the nutritional state has been observed also in several other insect species (Wigglesworth, 1936; Schwinck, 1951; Müssbichler, 1952; Engelmann, 1957).

In a general way, the effects of nutritional deficiency on the corpus allatum of the insects compare with those on its analogue, the anterior pituitary of the vertebrates.

A variety of studies in mammals have shown that, under conditions of starvation, (a) distinct structural and functional changes occur in the pars anterior, (b) gonadal malfunction is attributable to suppression of gonadotropic activity; and (c) the reproductive system deficient because of starvation responds to the administration of pituitary material.

The morphological changes observed in pituitaries of starving laboratory mammals as well as human patients, such as decrease in the volume or weight of the anterior lobe (Jackson, 1917), in the size and number of parenchymal (acidophilic) cells (Sedlezky, 1924; Schubothe, 1940; D'Angelo *et al.,* 1948), and in relative cytoplasmic content (Jackson, 1917), are in line with the changes described in the present study for the corpora allata of the insect, *Leucophaea.*

As in the insect, the concomitant disturbance of gonadotropic activity in the mammals studied led to depression of gonadal function (Mulinos and Pomerantz, 1940; Rinaldini, 1949/50) which could be remedied by the administration of gonad-stimulating substances (Boutwell *et al.,* 1948; Rinaldini, 1949/50).

It was of particular interest to observe the effects of the return to a normal diet in females of *Leucophaea* whose ovarian activity had been restrained by a 30-day starvation period following emergence. Instead of beginning their reproductive period in the same manner as young normal adults, even though belatedly, starved-re-fed females "skipped the first cycle" for which a slower and more moderate activation of the corpora allata is typical. At once they acted at full capacity. Thus, the return to ample food supplies may represent a powerful stimulus for corpus allatum growth. Within a short period of 20–25 days an up to seven-fold volumetric increase occurred. The subsequent decline from these high values was equally rapid. From every point of view, the curves illustrating this reproductive cycle do not differ essentially from those characteristic of the second or subsequent cycles in normal specimens (Figs. 2, 4). In fact, the peak illustrating the range of corpus allatum activation is higher in the starved-fed group than in any cycle of our normal series. However, this difference might perhaps be due to individual variation, since Engelmann's (1957) values for the second reproductive cycle of normal females reach approximately the same maximum as that obtained in the present series of experimental animals. On the other hand, the values obtained by Engelmann are not entirely comparable to those reported here, because his experiments were conducted under different conditions of temperature and humidity. At any rate, there can be no doubt that corpora allata of animals having been starved for some time and then returned to normal food supply, respond with great readiness and display pronounced signs of activation. The same observation was made in nymphs of *Panorpa* (Schwinck, 1951). In contrast to a newly emerged animal, a starved-fed female undergoing its first reproductive cycle has had time to adjust to the changes connected with "metamorphosis." This may be the reason why its corpora allata, like those of older normal specimens, respond maximally as soon as the restraining effect of the brain is lifted. This would mean that the "post-emergence maturation" postulated by Rockstein (1956) can take place under conditions of total starvation.

Be this as it may, within certain limits the degree of corpus allatum activation obtained under various normal and experimental conditions is perhaps not too significant. One must keep in mind that the more moderate activation of the corpora allata in the first normal cycle suffices for the development of a full set of eggs. The more pronounced response of the corpora allata in more mature adults, normal as well as starved-fed may be the result of a higher metabolic rate of their tissues. This possibility is suggested by comparable data in mammals. Here, re-feeding after starvation resulted in an increased metabolic rate (Quimby *et al.*, 1948) and in a prompt response of the anterior lobe (Jackson, 1917).

SUMMARY

1. Adult females of *Leucophaea maderae* were subjected to total starvation, with or without dehydration, following their emergence. Throughout the period of survival, *i.e.*, up to 95 days, no egg development occurred as a consequence of the failure of the corpora allata to become properly activated. The corpora allata of a series of starved animals fixed after varying intervals showed only a small initial rise in volume which was followed by a gradual decrease reaching a minimum below that of normal controls.

2. In another series of animals, starved for 30 days, the return to a normal diet promptly initiated a growth phase in the corpora allata which considerably surpassed that characteristic of the first reproductive cycle in normal animals (seven-fold, instead of four-fold volumetric increase). As to speed and degree, this period of activation compared favorably with that of the second cycle in normal females. Thus the delay in reproductive activity, caused by starvation, was at least in part compensated for by a more rapid and complete activation of the corpora allata which in turn promptly led to ovarian development.

3. The effects of starvation and re-feeding on the structure and function of the corpora allata of *Leucophaea* are in line with those described in the literature for the analogous organ in mammals, *i.e.*, the anterior lobe of the pituitary.

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SUBSTANCES WITH JUVENILE HORMONE ACTIVITY IN CRUSTACEA AND OTHER INVERTEBRATES[1]

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The cyclical growth and molting of immature insects is brought about by two hormones, one secreted by the insect's brain and the other by the prothoracic glands. A third hormone, the juvenile hormone, is secreted by the corpora allata, endocrine glands in the head or prothorax of the insect. This hormone promotes larval development but prevents metamorphosis (Wigglesworth, 1957). Its presence in the immature insect guarantees that when the larva molts it will retain its juvenile characters and not differentiate into an adult. The juvenile hormone is thus a remarkable molecule that permits growth but prevents maturation. So far as we are aware it has no functional counterpart in the vertebrates. Recently Williams (1956) has reported that ether extracts of the abdomens of male Cecropia moths (*Hyalophora cecropia* L.) contain large amounts of juvenile hormone. When this extract was injected into lepidopterous pupae, they molted into second pupae instead of molting into adults. This, of course, is precisely what occurs when active corpora allata are implanted into pupae (Piepho, 1951; Williams, 1952).

Although initial experiments demonstrated juvenile hormone only in extracts of male Cecropia moths, we have since extracted it from both males and females of 22 species of Lepidoptera representing 6 families (Schneiderman and Gilbert, 1957; Gilbert and Schneiderman, 1958a). This result suggested that the hormone could have a wider distribution in the animal kingdom. The experiments to be reported were conducted to determine whether substances with juvenile hormone activity could be extracted from other insect orders besides Lepidoptera, from other classes of arthropods and from other phyla.

MATERIALS AND METHODS

1. *Experimental animals*

Pupae of the polyphemus silkworm (*Antheraea polyphemus* Cram.) were used as test-objects for assay of juvenile hormone activity. They were stored for about thirty weeks at 6° C. prior to use.

2. *Preparation and assay of extracts*

Animals representing most of the major groups of invertebrates were collected at Woods Hole, preserved in methanol and shipped to Cornell University for ex-

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[1] This investigation was supported by grant H-1887 from the National Heart Institute of the U. S. Public Health Service.

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traction. Some animals (*e.g.,* earthworms and slugs) were collected locally and extracted immediately. The tissues were homogenized in ethyl ether and the homogenates and methanolic extracts vigorously re-extracted with ether in a continuous extractor. The ether extracts were washed several times with water, the ether evaporated off and the oily or waxy residue dried *in vacuo* at 60° C.

To test for juvenile hormone activity in the resulting extracts, many of which were toxic and waxy, a new and exceedingly sensitive assay procedure was developed which permitted detection of traces of juvenile hormone activity in crude extracts. The assay takes advantage of the extraordinary sensitivity of regenerating epidermal tissue to juvenile hormone (Piepho, 1950; Piepho and Heims, 1952). The extract to be assayed is mixed with peanut oil and paraffin wax. A small rectangle of integument is excised from the thorax of a Polyphemus pupa, a few crystals of streptomycin and phenylthiourea (an anti-tyrosinase) placed in the wound, and the wound sealed with a few milligrams of melted wax-peanut oil-extract mixture. When the adult moth emerges three to four weeks later, the wound area is examined. In the case of inactive extracts, the only evidence of the former wound is a small indentation covered with adult cuticle. However, if the extract is active, then an island of pupal cuticle occurs at the wound site. Figure 1 depicts such a patch of pupal cuticle. It stands out sharply from the adult cuticle which surrounds it. It is scale-less, brown, rugose and typically pupal in

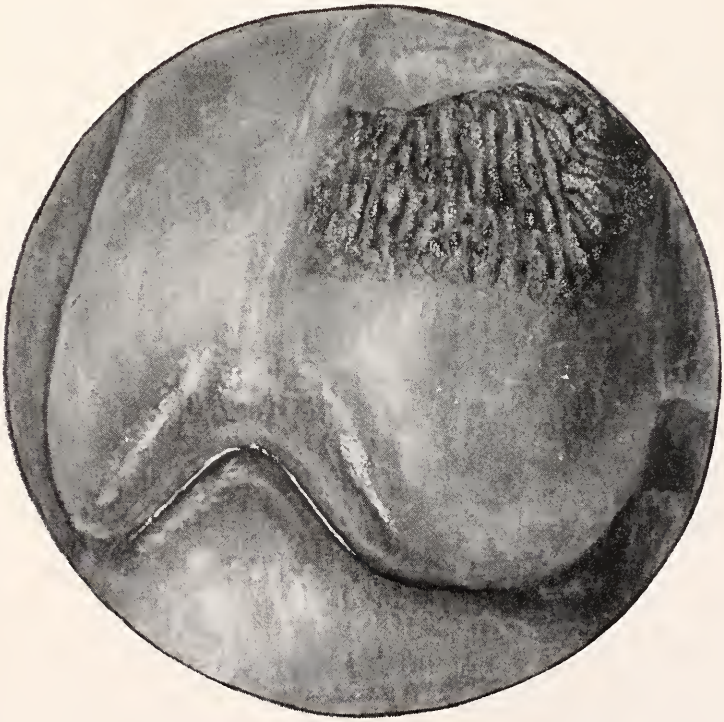

FIGURE 1. Thorax of adult Polyphemus with a pupal patch produced by the wax test. Scales have been removed.

TABLE I

Effects of serial dilutions of crude juvenile hormone extract in paraffin

Concentration of hormone in paraffin	Effect*		
0 (Peanut oil)	0	0	0
0 (Inactive oils)	0	0	0
0 (Paraffin)	0	0	0
1:2000	+	0	0
1:512	+	0	0
1:256	+	0	0
1:128	+	+	0
1:64	+	+	0
1:32	+	+	+
1:16	+	+	+
1:8	+	+	+
1:4	+	+	+
1:1	+	+	+

* Each symbol represents a test animal.

most other respects; it may even have pupal setae. In cross-section it appears to be three to four times as thick as adult thoracic cuticle. In short, it is essentially indistinguishable from ordinary pupal cuticle.

This "wax test" appears to be far more sensitive than other tests for juvenile hormone activity, as the following experiment reveals. A crude ether extract of the abdomens of male Cecropia moths was serially diluted with peanut oil (up to 1/1000) and these dilutions dissolved in equal parts of wax and applied to thoracic wounds as described above. The results recorded in Table I reveal that the assay permits detection of final dilutions of hormone of 1/2000. That is, in principle, an extract that contained only 1/1000th as much juvenile hormone activity as male Cecropia extract would yield a positive result. In the 1/2000 dilution recorded in Table I, the wax patch weighed about 6 milligrams and, therefore, contained only *3 micrograms* of crude extract. Hence it is possible with this test to assay the juvenile hormone content of minute quantities of material extracted from a part of a single insect.

It is important to note that, so far as we can ascertain, the wax test is absolutely specific for juvenile hormone activity. Thus wax alone, or mixtures of wax with peanut oil, have never given us false "positive tests" although dozens of control tests have been made. Moreover, when the active principle is removed from the crude extract by repeated liquid-liquid extractions, the oil that remains, containing virtually all of the ether-extractable material in the original extract, is also inactive in the wax test.

RESULTS AND DISCUSSION

Using this sensitive test, extracts from 13 classes of invertebrates representing most of the major phyla were examined. The results presented in Table II reveal that ether extracts of a truly diverse array of invertebrates possess at least some juvenile hormone activity. It is not too surprising to find juvenile hormone activity in crustaceans and even in annelids, but surely its presence in hydroids and sea cucumbers is unexpected.

TABLE II

Juvenile hormone activity of ether extracts of various invertebrates

Phylum	Class	Species	Wax test
Porifera	Demospongiae	*Microciona prolifera*	0
		Cliona celata	0
Cnidaria	Hydrozoa	*Pennaria tiarella*	+
		Tubularia crocea	+
	Anthozoa	*Metridium dianthus*	0
Rhynchocoela	Anopla	*Cerebratulus* sp.	
		(bodies)	0
		(heads)	0
Annelida	Polychaeta	*Nereis virens*	
		(bodies)	+
		(heads)	+
	Oligochaeta	*Lumbricus terrestris*	
		(bodies)	+
		(heads)	+
Arthropoda	Insecta	Numerous Lepidoptera	+
		Tenebrio molitor (Coleoptera)	
		(larvae)*	+
		(adults)	0
		Sarcophaga bullata (Diptera)	
		(larvae)	0
		Neodiprion lecontei (Hymenoptera)	
		(diapausing prepupae)	0
		Apis mellifera (Hymenoptera)	
		(winter workers)	0
	Crustacea	*Uca pugilator*	+
	(Decapoda)	*Orconectes immunis*	
		(entire)	0
		(purified extract)	0
		Homarus americanus	
		(eyestalks)	+
		Carcinides maenas	
		(fronts)	0
		(rears)	0
		Palaemonetes vulgaris	0
	Arachnida	*Limulus polyphemus*	
		(fronts)	0
		(rears)	0
		(purified sterols)	0
Mollusca	Gastropoda	*Deroceras* (*Agriolimax*) *agreste*	
		(heads)	0
Echinodermata	Holothuroidea	*Thyone briareus*	0
		Leptosynapta inhaerens	+
	Echinoidea	*Arbacia punctulata*	0
Enteropneusta	Balanoglossida	*Saccoglossus kowalevskii*	
		(entire)	+
		(less collar and proboscis)	+

* Tested by injecting extract.

It is of some interest that the most potent non-insect extract came from the eyestalks of lobsters. The occurrence of high concentrations of substances with juvenile hormone activity in the eyestalk, which is a well-known endocrine center in crustaceans (Knowles and Carlisle, 1956), suggests that in crustaceans the eyestalk may contain a gland which produces a substance chemically similar to the juvenile hormone of the corpora allata. A likely site is a part of the X-organ which is not neurosecretory but appears glandular (*i.e.*, the secretory cells of the sensory papilla X-organ (Knowles and Carlisle, 1956)). Whether the juvenile hormone plays a role in crustacean development or egg maturation remains to be proved, but it appears likely. In addition to these results we have also recently found juvenile hormone activity in the adrenal cortex of cattle (Gilbert and Schneiderman, 1958b). Hence, it seems safe to conclude that substances with juvenile hormone activity are widespread in the animal kingdom. As far as we are aware, the only other animal growth hormones of such wide distribution are the estrogens (Loewe *et al.*, 1932; Hagerman *et al.*, 1957).

Whether or not these juvenile hormone substances are similar chemically to the juvenile hormone of insects cannot be answered until the structure of the juvenile hormone is known, nor do we know at present what role these juvenile hormone substances play in groups other than insects. Nevertheless, it remains an intriguing fact that substances that act as a growth hormone for insects occur in both hydroids and cattle. It supports the view that in the course of evolution there have not been a great number of innovations at the level of small molecules since the Cambrian Era, and that the evolution of humoral mechanisms has proceeded by particular groups of animals adapting available and often ubiquitous molecules to special tasks.

We wish to thank Dr. Berta Scharrer for critical reading of the manuscript of the present paper. Purified *Limulus* sterols were generously supplied by Dr. Werner Bergmann.

SUMMARY

1. A new assay for the juvenile hormone of insects is described which permits detection of very small amounts of hormone activity.

2. Using this procedure extracts of a variety of invertebrates were assayed for juvenile hormone activity.

3. Juvenile hormone activity was detected in Hydrozoa, Polychaeta, Oligochaeta, Lepidoptera, Coleoptera, Decapoda, Holothuroidea, and Balanoglossida.

4. The richest source of juvenile hormone outside of insects was the eyestalk of Crustacea and it is suggested that the juvenile hormone plays a role in crustacean physiology.

5. The significance of these findings is discussed in relation to the evolution of humoral mechanisms.

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A STUDY OF SOME EFFECTS OF GAMMA RADIATION ON THE ADULTS AND EGGS OF AEDES AEGYPTI

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The studies to be reported in this paper were undertaken to evaluate some of the biological effects of ionizing radiation on the mosquito *Aedes aegypti*. This phase of the work describes in particular the effects produced by gamma radiation on the fertility and reproductive capacities of the males and females of this mosquito species, detailing as well, additional studies on the effects of radiation on the viability of eggs in various stages of development or age. It may be of interest to note that although there is an extensive literature on irradiation of insects, and in particular on irradiation of *Drosophila*, nevertheless, these studies have been concerned in the main with genetic effects rather than with the specific biological effects reported here.

Materials and Methods

The strain of *A. aegypti* used in these experiments was obtained originally from the U. S. Bureau of Entomology and Plant Quarantine Laboratory of Beltsville, Maryland, in June, 1945. It has been maintained since that time in this laboratory in wire screened cages, measuring 27 × 24 ×24 inches, at a constant laboratory temperature of 80° F. and a relative humidity of 75 per cent. To maintain egg production the females have been allowed to gorge on guinea pigs once a week. The resulting eggs have been collected on strips of filter paper, and then conditioned by storing the wet strips in closed jars for a period of three days, after which they are dried and stored at room temperature for future use. The larvae from hatched eggs have been reared in glass jars containing approximately 2500 ml. of tap water, and have been fed on guinea pig pellets added in appropriate amounts each day. On this regimen, it usually requires 8 days for newly hatched larvae to reach the pupal stage. All the adults and eggs exposed to gamma radiation were derived from this colony.

The adults used for experimental purposes were kept in plastic cylinders of 3 inches height and 4 inches diameter, in groups of 40 males and/or 40 females, and were fed during the course of the experiments on 4 per cent sugar solutions. Experimental groups which require blood were fed exclusively on chicks, usually weighing about 300 grams. Adult mosquitoes were kept in the same cylinders during exposure to radiation, and it was possible to expose 6 cylinders simultaneously in the cobalt 60 irradiator used for these experiments. The strips of paper holding the eggs were placed in Petri dishes, and following exposure to radiation were stored in the same dishes. To insure a maximal hatch, irradiated eggs were kept immersed in water for at least 72 hours.

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[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

The mosquitoes were exposed to gamma radiation at an approximate rate of 450 r per minute in air, in a cobalt 60 irradiator of 4 pi geometry.

RESULTS

Irradiation of the male

In the first series of experiments designed to determine the effects of varying dosages of gamma radiation on male *A. aegypti,* 40 male and 40 female mosquitoes were allowed to emerge separately into plastic cylinders and on the fourth day following emergence, the males were exposed to gamma radiation in the cobalt irradiator. On either the first, the eighth, or the fifteenth day following exposure to radiation, the males were then placed in the cylinders containing the females, and shortly after pairing the females were allowed to gorge themselves on normal chicks. The paired mosquitoes then remained together for the duration of the experiment. Control groups were handled in exactly the same manner except that they, of course, received no radiation.

At each dosage level, two to four trials were conducted for both the experimental and control groups, and the number of eggs laid by each group and the percentage of the eggs which hatched were estimated as accurately as possible. Following hatching, the larvae from the experimental groups were grown in the usual manner to determine whether or not they would develop into viable adults. If adults finally developed, these, in turn, were given a blood meal and allowed to mate and the resulting eggs were immersed in water as a final test of parental fertility. Finally, many of the male mosquitoes were dissected at suitable intervals following exposure to radiation in order to determine the presence and condition of the spermatozoa in the seminal vesicles. At the same time, non-irradiated females which had been paired with irradiated males were also dissected to determine the presence and condition of spermatozoa within the spermathecae.

Table I summarizes most of the data concerning the effects of gamma radiation on male *A. aegypti.* In dosages ranging from 1000 r to 30,000 r, exposure to radiation produced no significant effect on the number of eggs laid in those groups in which the males were irradiated one day before they were mated to normal females. Both the control, or non-irradiated animals, and the experimental ones deposited about the same total number of eggs. In the groups in which the males were irradiated but were not paired and offered a blood meal until 8 days after radiation, however, there was evidence of some reduction in the number of eggs deposited among those in which the males had received 30,000 r, while among the groups paired and given a blood meal 15 days after the males had been irradiated, it required only 20,000 r to produce a significant reduction in the number of eggs deposited by the females. In these groups the pattern of egg-laying activity resembled that of 5-day-old and 19-day-old virgin females, allowed to take a blood meal. Such non-inseminated females produced about 20 per cent less the number of eggs than that produced by inseminated females of comparable age, and in addition, the oviposition period of such females extended over significantly longer periods of time.

Table I

The oviposition pattern of normal female Aedes aegypti mated to males exposed to varying doses of gamma radiation

Dosage (r)	Days post-irradiation, mating and blood-meal								
	1			8			15		
	No. eggs laid	Length oviposition period (days)	Per cent hatch	No. eggs laid	Length oviposition period (days)	Per cent hatch	No. eggs laid	Length oviposition period (days)	Per cent hatch
0	1060	11	100	1250	13	100	1100	16	100
1,000	1440	12	84	900	10	95	950	9	98
2,500	940	10	64	1150	12	85	1400	13	46
3,500	880	14	43	1000	13	46	1200	17	38
5,000	1150	12	5	1050	13	8	1600	26	9
7,500	1110	9	5*	1230	11	6*	1400	21	2*
10,000	1530	14	1	1000	14	2	1150	25	2
20,000	900	14	0	1100	22	0	800	41	0
30,000	1000	11	0	850	40	0	750	57	0

* Fertile F-1 progeny reared.

The length of the oviposition period was markedly affected by both the quantity of radiation applied to the males and the interval between exposure to radiation and mating. In 27 control groups given a blood meal and allowed to mate on the fifth day following emergence, eggs were deposited for periods ranging from 8 to 15 days, averaging 11 days, after the blood meal. In 27 corresponding experimental groups, in which males exposed to doses of radiation ranging from 1000 r to 30,000 r on the fourth day following emergence were mated on the next day and the females then given a blood meal, the length of the oviposition period differed very little from that of the control animals. In a second series, in which the females of 25 control groups were given a blood meal and allowed to mate on the twelfth post-emergence day, eggs were deposited for periods ranging from 8 to 16 days, averaging 13 days, after the blood meal. In the corresponding experimental groups, in which the males were irradiated on the fourth post-emergence day and mated 8 days later, there was no significant difference in the length of the oviposition period among those groups in which the males received radiation dosages ranging up to 10,000 r. At dosages of 20,000 r, however, eggs were deposited up until 22 days following the blood meal, and in the groups in which the males had received 30,000 r, the females continued to deposit eggs for periods as long as 40 days after the blood meal.

Increases in length of the oviposition period were even more apparent in the third set of experimental groups, in which the males and females were paired and the females then given a blood meal 14 days after the males had been exposed to radiation. In this series, 24 control groups in which non-irradiated mosquitoes were mated and given a blood meal on the nineteenth day after emergence, eggs were deposited for periods ranging from 9 to 25 days, averaging 16 days, after the blood meal. In the experimental groups in which the males had received radiation dosages up to 3500 r on the fourth day and mated 15 days later with normal females, the length of the oviposition period was similar to that of the control groups cited above. At dosages of 5000 r to 10,000 r, however, eggs were deposited for periods which averaged 24 days after the blood meal, while at 20,000 r dosages, this period was extended to an average of 41 days, until at 30,000 r dosages the females continued to lay eggs for periods averaging 57 days. It is of interest to note that along with the increase in the egg-laying period, there was a corresponding increase in the number of days in which the females laid eggs. Thus, in the series in which males exposed to 30,000 r were mated to normal females 8 days later, and in which the oviposition period was extended to 40 days, eggs were actually laid during this interval on 27 different days as compared to 9 days on which normal females mated to normal males laid eggs. In the most extreme case, in which males exposed to 30,000 r were mated 15 days later, the females laid eggs on 33 different days as compared to 10 days for the control females in that series.

In view of these results, it was necessary to determine, first, the effects of gamma radiation on the spermatozoa in the male mosquito. For this purpose, males were dissected and the seminal vesicles examined at appropriate intervals following exposure to varying dosages of radiation. At dosages up to 10,000 r, there was no evident effect on motility, nor any evidence of morphological damage to the spermatozoa for at least 40 days following exposure to radiation. Similarly, at dosages of 20,000 r there was no evidence of loss of motility or morphological damage to the spermatozoa for a period of 25 days. After 25 days, however, during which period the mosquitoes began dying from the effects of the radiation, the spermatozoa were found to be in various stages of fragmentation and degeneration. In males exposed to 30,000 r, loss of motility and deterioration of the spermatozoa did not occur until 20 days after irradiation, at which time again the mosquitoes began dying from the effects of the irradiation. With the radiation dosage increased to 50,000 r, spermatozoa remained motile and normal in appearance for only about 10 days, at which time deterioration of the spermatozoa and death of the adults began to occur simultaneously as usual. In generai, therefore, spermatozoa remained normal in appearance and motile for about as long as the mosquitoes themselves were able to survive the various doses of radiation.

Secondly, to determine whether insemination had occurred, and to observe the condition of the spermatozoa in inseminated females, the spermathecae of normal females mated to irradiated males were examined at appropriate intervals after the animals had been paired. As a result, it was found that all the females paired with males one day after the males had been exposed to 20,000 r contained motile spermatozoa normal in appearance for at least two weeks after mating. On the other hand, only about one-half the females mated 15 days after the males had been exposed to 20,000 r contained normal spermatozoa the third day after mating. In the remainder, either the spermatozoa were in fragments or, more usually, there were no spermatozoa present. And again, the spermathecae of all the females mated a day after the males had been exposed to 30,000 r contained normal appearing spermatozoa for at least two weeks after pairing. If, however, pairing was delayed until 15 days after exposure of the males to radiation, none of the spermathecae of the 25 specimens were found to contain spermatozoa when examined the day after mating. Thus, these data suggest that the reduction in egg production and increase in the period of oviposition evidenced when mating was delayed following exposure of the males to radiation was due simply to inability of more and more of the deteriorating males to copulate successfully rather than to any lack of motile spermatozoa.

That morphological integrity and motility of the spermatozoa, or significant changes in the egg-laying pattern of females mated to irradiated male mosquitoes, are inadequate criteria for determining the biological effects of gamma radiation on the fertility of the males, is shown by observations on the hatching of eggs laid by normal females mated to males exposed to varying doses of radiation, and the viability of the larvae emerging from such eggs. Thus, as shown in Table I, although in all the groups mated at various intervals following irradiation of the males, the females continued to lay eggs in considerable numbers, nevertheless, the number of larvae hatching from these eggs become progressively less with increasing exposure to irradiation of the males.

In view of the fact that even under well controlled conditions of temperature and humidity, there was considerable variation in the percentage of eggs hatching from eggs produced from normal matings, it was necessary for purposes of comparison, to consider the hatch from a given number of normal, control eggs as a 100 per cent hatch. And again because of variations in the hatch from a given collection of eggs, differences in the degree of hatch between eggs from control and experimental were not too apparent until the disparity in hatching between the two groups became quite considerable.

As shown in Table I, in all three groups mated at various intervals following irradiation of the males, the first real evidence of any appreciable reduction in the percentage of larvae hatching from a given number of eggs was in those groups in which the males were exposed to at least 2500 r. In the groups in which the males had received 3500 r there was little further reduction in hatch except in the group in which the males were not mated with normal females until 15 days after their exposure to radiation, in which case the hatch was only 18.4 per cent of that in the control group. There was, however, a sharp decrease in the hatch from eggs derived from groups in which the males had received 5000 r, so that in the ones mated 1 and 8 days following exposure to radiation, only 5.3 per cent and 7.6 per cent, respectively, of the eggs hatched, as compared to the controls. On the other hand, as if to illustrate again the variations in hatching which may occur, in the third remaining group which had been mated 15 days following exposure to radiation, 20.0 per cent of the eggs produced hatched into viable larvae.

There was little further reduction in the proportion of eggs hatching among the groups in which the males had received 7500 r. In the groups in which the males had been exposed to 10,000 r, however, only slightly over 1 per cent of the eggs hatched, while in the groups in which the males had received 20,000 r and 30,000 r, no larvae ever hatched from the eggs which were produced.

The fact that the number of viable eggs produced was the same, at any given dosage, in the groups mated 15 days after irradiation as in the groups mated the day following exposure, indicates that once a sperm was damaged to the extent that it was no longer capable of fertilizing an egg, there was no further recovery and the injury remained permanent, whereas if a sperm escaped such lethal injury initially there was no further physiological deterioration and it remained uninjured and capable of fertilizing an egg. Since there was no decrease or increase in the production of viable eggs to indicate either deterioration or recovery, neither of which process could be expected to proceed at the same rate, it appears reasonable to assume that gamma irradiation has a kind of all-or-none effect on the spermatozoa of *A. aegypti,* and that the extent of this effect will depend upon the level of radiation that has been administered. Further, the data cited above lend further support to the assumption that spermatogenesis is not a continuing process during the adult life of the male *A. aegypti.*

It is of interest to note that the larvae hatching from the matings described above could be reared successfully and the resulting adults, when mated themselves, produced fertile, viable eggs, providing there were sufficient larvae present to eliminate the cultivation problems that arise when larval colonies are too small. Thus, from matings in which the males had received dosages up to 3500 r, larvae were reared with comparative ease, but at dosages of 5000 r and 7500 r, when only very few larvae were available, rearing them to adulthood became a major problem of cultivation. Even at these dosages, however, the few larvae that were finally grown mated successfully as adults and produced viable progeny. Although several attempts to rear the isolated larvae hatching from matings in which the males had received 10,000 r were unsuccessful, there is some reason to believe that with enough care such larvae could be grown to adults, and in such a case the adults would in all likelihood produce viable progeny.

Irradiation of the females

In these experiments, designed to measure the effects of varying doses of gamma radiation on the oviposition habits of female *A. aegypti,* again 40 male and 40 female mosquitoes were allowed to emerge separately into plastic cylinders except that in this case on the fourth day following emergence, the females were irradiated and then paired with normal male mosquitoes on the first, eighth, or fifteenth day following exposure to radiation and offered a blood meal soon after they had been mated. The resulting eggs were collected, counted, then incubated as usual in a saturated atmosphere for five days, and finally hatched. The larvae were grown to adults and mated, and the resulting eggs were then collected and allowed to hatch as evidence of fertility of the F-1 adults. It may be noted here that as in the case of eggs produced from matings in which the males were irradiated, if the eggs hatched they could usually be grown to adults.

As shown in Table II, there was no significant reduction in the number of eggs produced by females exposed to 1000 r, or 2000 r, and mated the following day. There was, however, a significant reduction in the number of eggs produced by females exposed to 2500 r, while the females exposed to doses as high as 10,000 r produced only a few isolated eggs. On the other hand, in the groups mated 8 and 15 days following irradiation, although again there was little or no reduction in the number of eggs produced by females exposed to doses up to 2000 r, egg production dropped off sharply in females exposed to 2500 r, while females exposed to 5000 r laid no eggs at all. In general, therefore, the longer the mating of females exposed to radiation doses above 2000 r was delayed, the fewer the eggs they produced, and the lower the dosage required to eliminate egg production entirely. Similarly, the fewer the eggs that were produced, the less the proportion of them that eventually hatched into viable larvae. Thus, whereas 64.9 per cent of the eggs laid by females exposed to 3000 r and mated one day later hatched, only 25.6 per cent of the eggs laid by females exposed to the same dosage but mated 15 days later hatched, and similarly while 67.0 per cent of the eggs laid by females exposed to 3500 r hatched when they were mated 24 hours later, only 24.0 per cent of the eggs hatched when mating was delayed 15 days. And, finally, although a small percentage of the eggs laid by females exposed to 5000 r and mated one day later hatched into viable larvae, none of the females exposed to 5000 r or higher laid any eggs at all when mated 8 or 15 days following exposure.

These data indicate that in female *A. aegypti* exposed to radiation above certain threshold levels, in this case approximately 2000 r, the ovaries are not only incapable of recovering from the injury produced by radiation, but rather that the functional activity of the ovary becomes progressively further impaired during the interval following exposure. It may be noted in passing that unlike the case of normal females mated to males exposed to radiation, in which the reduction in egg production resulted in lengthening of the period of oviposition, in this case the reduced egg production of irradiated females mated to normal males resulted instead in significant lessening of the oviposition period, indicating again the loss in functional activity of the impaired ovaries.

To determine whether irradiated females could mate successfully and whether spermatozoa could survive in them, groups of virgin females were exposed to doses of 20,000 r and 30,000 r, paired with normal males either 1 or 15 days later, and then dissected at suitable intervals following mating. The spermathecae of all the females exposed to 20,000 r, and paired the day following radiation, contained motile spermatozoa for a period of two weeks after mating had occurred. However, when

TABLE II

The oviposition pattern of female Aedes aegypti exposed to varying doses of gamma radiation and then mated, at different intervals following irradiation, to normal males

Dosage (r)	Days post-irradiation, mating and blood meal					
	1		8		15	
	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch
0	1130	100	1070	100	1100	100
1,000	1050	100	1100	100	1000	100
1,500	1350	71	1050	90	1000	91
2,000	1000	87	1000	90	700	78
2,500	700	87	830	62	650	32
3,000	650	65	800	42	400	26
3,500	550	43	600	35*	190	24*
5,000	25	13*	0		0	
7,500	10	0				
10,000	10	0				
20,000	0					

* Fertile F-1 progeny reared.

mating was delayed until 15 days after irradiation, an average of only 8 out of 10 females were found to contain spermatozoa during the ensuing two weeks. Similarly, the spermathecae of all females exposed to 30,000 r contained spermatozoa for at least 2 weeks if pairing took place the day after irradiation, but if mating was delayed until 15 days after exposure to radiation, then only an average of 4 out of 10 females dissected at intervals during the subsequent 2 weeks contained spermatozoa. From these results, it would appear as if females exposed to high doses of radiation are capable of mating shortly after exposure and that spermatozoa will apparently survive in them for at least two weeks, but if mating is delayed too long, radiation injury to the mosquito as a whole progressively reduces the chances of successful mating

The second series of experiments was designed to ascertain whether viability or production of eggs was influenced by insemination of the females prior to radiation rather than subsequent to radiation as in the previous experiments. Accordingly, to assure insemination 40 females and 40 males were allowed to emerge into the same cylinders and were maintained together for four days following emergence. At the end of this period the males were removed and the females were exposed to varying doses of radiation. One, eight and fifteen days after irradiation these females were allowed to take a blood meal and the resulting eggs were collected, incubated and hatched. The data presented in Table III indicate that females inseminated prior to irradiation and given blood at various subsequent intervals laid about the same number of eggs as females inseminated at similar intervals following exposure to radiation, as shown previously in Table II. Similarly, as in the previous experiment, the number of eggs produced decreased significantly at

Table III

The oviposition pattern of female Aedes aegypti mated first to normal males, exposed to varying doses of gamma radiation after mating, and then allowed to take a blood meal at different intervals following irradiation

Dosage (r)	Days post-irradiation, blood meal					
	1		8		15	
	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch
0	1300	100	1280	100	1000	100
1,000	1400	75	1300	68	1250	86
1,500	1450	55	1220	68	1320	60
2,000	1350	67	1150	61	900	57
2,500	1180	67	910	33	630	44
3,000	600	34	620	15*	670	14
3,500	380	3	200	0	520	7*
5,000	30	2*	0		0	
7,500	30	0	0		0	
10,000	40	0				
20,000	0					

* Fertile F-1 progeny reared.

2500 r to 3000 r, and except for those females which received a blood meal one day after irradiation, egg-laying was almost entirely inhibited at doses 5000 r or above.

On the other hand, the number of eggs which hatched from among those laid by females inseminated prior to exposure to radiation was consistently below the number which hatched from among those laid by females inseminated subsequent to exposure to radiation. Thus, in the groups in which the females were mated and then exposed to as little as 1000 r there was a significant reduction in the number of eggs hatching from among those produced, while there was no reduction in the number of eggs that hatched of those produced by females inseminated following exposure to the same dosage, whereas 43 per cent of the eggs laid by females inseminated subsequent to exposure to 3500 r hatched out, only 3 per cent of the eggs produced by females inseminated prior to exposure hatched into viable larvae. Obviously, insemination of females prior to exposure to radiation reduces the viability of the eggs simply by introducing an additional source of injury, namely, injury to the spermatozoa in addition to the effect on the ovaries.

As before, it was possible to rear fertile F-1 progeny whenever enough larvae hatched out from the eggs. Thus, fertile F-1 progeny were reared from females inseminated either before or after exposure to doses as high as 5000 r and then given a blood meal the day after radiation. At dosages above 5000 r although a few eggs were produced, as shown in Tables II and III, none hatched. On the other hand, no eggs were produced by females exposed to 5000 r but not allowed a blood meal until 8 and 15 days following exposure to radiation, and fertile progeny were reared only from females receiving 3500 r.

In female *A. aegypti* ovarian activity is apparently suspended until the animal takes a blood meal. Following a blood meal development of the egg proceeds, fertilization takes place providing the spermathecae contain spermatozoa, and finally oviposition begins. At the temperatures maintained in this laboratory, female *A. aegypti* will begin to produce fertile eggs approximately 48 hours after a blood meal has been taken.

The next series of experiments was designed to study the effects of radiation on the cycle of events occurring in fertilized females between the time blood is ingested and oviposition begins. Thus, in these experiments again 40 males and 40 females were allowed to remain together for 4 days following emergence, then at the conclusion of this period, the males were removed and the females were given a blood meal. Subsequently, at intervals of 4, 24 and 42 hours after the blood meal the females were exposed to varying doses of radiation.

The results shown in Table IV indicate that the various doses of radiation had their greatest effect on egg production in those groups irradiated 4 hours after a blood meal, a significantly lesser effect in the groups irradiated 20 hours later, and the least effect in the groups irradiated 42 hours after the blood meal. Thus, whereas the group exposed to radiation 4 hours after it had received a blood meal laid a total of 2305 eggs and no eggs were produced by any females exposed to more than 10,000 r, the group exposed 24 hours after it had engorged laid 12,610 eggs and a few eggs were produced by females receiving as much as 70,000 r. Finally, in the group irradiated 42 hours after the blood meal, 11,010 eggs were laid and it required exposures in excess of 100,000 r to inhibit egg production com-

Table IV

Oviposition and egg viability of inseminated female Aedes aegypti exposed to gamma radiation at various intervals following a blood meal

Dosage (r)	Hours post-blood-meal. irradiation					
	4		24		42	
	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch	No. eggs laid	Per cent hatch
0	1200	100	1400	100	1480	100
2,500	1060	69	1380	45	1270	29
3,000	600	53	1400	38	1400	13
3,500	230	31	1660	30	1340	7*
5,000	160	7*	1590	10*	1280	1
7,500	110	8	1720	6	1480	0
10,000	40	0	1970	0	1180	0
20,000	0		970	0	980	0
70,000			10	0	310	0
80,000			0		130	0
100,000					10	0
110,000					0	

* Fertile F-1 progeny reared.

pletely. It would appear from these data that the early phases of the complicated sequence of physiological events leading to egg production are highly sensitive to radiation injury but that once the mechanism has been established and has proceeded to some specific developmental stage, oviposition will take place in spite of excessive radiation injury.

On the other hand, the data show that although the mechanisms responsible for egg production become more resistant as development proceeds, the eggs themselves become more sensitive to the effects of radiation as they mature during the pre-oviposition period. Thus, in the groups irradiated 4 and 24 hours following the blood meal, about 8 and 6 per cent, respectively, of the eggs produced by the females exposed to 7500 r hatched into viable larvae but in the groups irradiated 42 hours after the blood meal, only about 1 per cent of the eggs produced by females exposed to 5000 r hatched, while none of the eggs produced by females exposed to 7500 r proved to be viable. And similarly, in the lower dosages, the hatch from eggs produced by females exposed to radiation 4 hours after the blood meal was far more abundant than the hatch from eggs produced by females exposed 42 hours later.

Viable, fertile F-1 progeny were obtained from the eggs produced by females exposed to 5000 r 4 and 24 hours after the blood meal, but the few larvae from eggs of females exposed to 6000 r and 7500 r died shortly before the pupal stage. In the groups irradiated 42 hours following the blood meal, viable, fertile F-1 progeny were reared from the larvae that hatched from eggs deposited by females exposed to 3500 r, but again the few larvae hatching from eggs deposited by females exposed to 5000 r died during cultivation. There is reason to believe that with greater numbers of larvae available, it might have been possible to rear adults from the groups in this experiment in which the larvae died during cultivation. Nevertheless, as before, whenever larvae from irradiated parents could be grown to adulthood, the progeny always proved to be fertile.

Irradiation of eggs

In the first experiments designed to determine the effects of ionizing radiation on the eggs of *A. aegypti,* eggs of two different age groups were selected for study. The first group consisted of eggs that were 25 to 50 hours old in which embryonation had not been completed, while the second group consisted of eggs approximately 400 hours old in which such development had long been completed so that the eggs would normally hatch at once upon immersion in water. In this laboratory, properly conditioned, normal eggs usually require 65 hours from the time they are laid until they hatch. Since the dose rate was about 450 r per minute, the eggs in the first groups were in the cobalt irradiator for periods not above 100 minutes, but the eggs of the second group, which required extraordinarily high doses of radiation to inhibit hatching, had to be kept in the cobalt irradiator for periods up to about 23 hours. Following exposure to radiation, the eggs were kept at insectary temperatures for 1, 8, or 15 days before being allowed to hatch. About 2000 eggs were used for each exposure and there were from 4 to 8 different exposures for each age category. To assure hatching, if hatching was to occur at all, eggs were kept immersed for 72 hours whenever necessary.

The data presented in Table V indicate that the hatching capabilities of the eggs which were 25 to 50 hours old when irradiated, and which have been designated as 2 days old, were far more susceptible to radiation injury than the eggs which were 16 days old when they were irradiated. Thus, whereas in the former it required only 10,000 r to reduce the hatch by 50 per cent, in the latter it required dosages ranging from 20,000 r to 100,000 r to reduce hatching to the same extent. And again, while a radiation dose of 20,000 r sufficed to inhibit completely

TABLE V

The effect of gamma radiation on the hatch of 2-day-old and 16-day-old Aedes aegypti embryos

Dosage (r)	2-day-old eggs (25–50 hrs.) Days post-irradiation, per cent hatch			16-day-old eggs Days post-irradiation, per cent hatch		
	1	8	15	1	8	15
2,500	100	100	100	100	100	100
5,000	100	100	80	100	100	80
7,500	75	75	75	100	80	80
10,000	50	50	50	80	75	75
15,000	25	25	25	80	75	75
20,000	0	0	0	80	75	50
30,000				75	50	25
100,000				50	20	10
150,000				25	0	0
200,000				10		
500,000				10		
550,000				0		

the hatching of 2-day-old eggs, which were being exposed while embryonic development was still in progress, it required doses ranging from 150,000 r to as much as 550,000 to eliminate entirely the hatching of 16-day-old eggs in which, of course, embryonic development had been completed prior to exposure. In addition, as shown in Table V, it was found that storage of 2-day-old eggs following exposure to radiation produced no further deterioration in the ability of the eggs to hatch but that storage of 16-day-old irradiated eggs resulted in marked deterioration, at the various dosage levels, in the ability of the eggs to hatch successfully. Thus, in 2-day-old eggs, as many eggs hatched out when immersed 15 days subsequent to doses of 10,000 r and 20,000 r as had hatched following immersion only one day following exposure, but with 16-day-old eggs, none of the eggs exposed to 150,000 r hatched out at the end of 15 days although 25 per cent of them had hatched when immersed the day following exposure. And again, although 10 per cent of the eggs which had received 500,000 r hatched when immersed the day following exposure, a comparable hatch was obtained from eggs which had received only 100,000 r when immersion was delayed 15 days following exposure to radiation.

It is of interest to note that although it required enormous doses of radiation to destroy the ability of the larvae to hatch, and there were wide differences between 2-day-old and 16-day-old eggs in the amount of radiation required to produce this effect, nevertheless, fertile F-1 adults could not be produced from either 2-day-old or 16-day-old eggs exposed to more than 2000 r, whether hatched 1, 8, or 15 days following exposure. Thus, only about 50 per cent of the larvae from either 2-day-old or 16-day-old eggs exposed to 1000 r developed into adults, almost all of which, however, were able to mate successfully. From eggs exposed to 1500 r, no more than about 10 per cent of the larvae developed into adults, and of these probably one-half were able to mate. From eggs exposed to 2000 r only about one per cent of the larvae developed into adults, and of these approximately one-third were able to mate and produce viable eggs. The remaining adults in these groups were usually too feeble even to feed and most of them died almost immediately after emergence. However, all the males examined in such cases were found to contain motile spermatozoa. It may be noted, too, that a considerable proportion of the mortality in these groups occurred after ecdysis when the animals, apparently too weak to fly off properly, simply fell back into the water. The larvae developing from eggs exposed to 2500 r died during either the fourth larval instar or the pupal stage, while very few larvae from eggs exposed to 3000 r survived beyond the third instar, but of those that did, all died during the fourth larval stage.

Finally, experiments were designed to determine the effects of varying doses of radiation on the hatching ability of eggs of *A. aegypti* of various ages. For this purpose the eggs were kept at room temperature for periods ranging from less than 24 hours up to 180 days, exposed to radiation and then immersed for hatching 4 days after having been irradiated. Immersion had to be delayed 4 days in order to insure embryonation of the eggs which were irradiated while they were still less than 65 hours old. About 2000 eggs were used in each trial and every group of irradiated eggs was matched by a control, or non-radiated group of eggs from the same adult colony. The experiment was terminated with 180-day-old eggs, since it was found that the hatching of control eggs was so poor after that period that it was almost impossible to isolate any effect of radiation. No hatch whatsoever could be obtained from 270-day-old control eggs.

It is evident from the data presented in Figure 1 that resistance or susceptibility to radiation was related to the age of the egg. During the first 24-hour period following oviposition, a time of active embryonic development, the eggs were particularly susceptible to radiation damage, so that as little as 800 r caused a 50 per cent reduction in hatch while 6000 r inhibited hatching entirely. By the time the eggs were 48 hours old and embryonic development was nearly complete, however, their resistance had increased so markedly that it required 7500 r to produce a 50 per cent reduction in hatch and 25,000 r to eliminate hatching entirely. During the period in which hatching would have normally occurred (third day), resistance was still further increased so that it required 30,000 r to effect a 50 per cent reduction in hatch. Resistance to radiation increased to maximal dosage levels in four- and five-day-old eggs so that it required 75,000 r to produce a 50 per cent reduction in hatch, and at least 130,000 r to inhibit hatching completely. Further aging gradually lessened resistance, however, until by the 180th day after oviposition, administration of 4000 r reduced the hatch by 50 per cent although it still required some 75,000 r to eliminate hatching completely. Progeny were reared from eggs of various ages irradiated at dosages ranging from 1000 r to 2500 r and there was no discernible evidence to indicate that the age of the egg influenced in any way

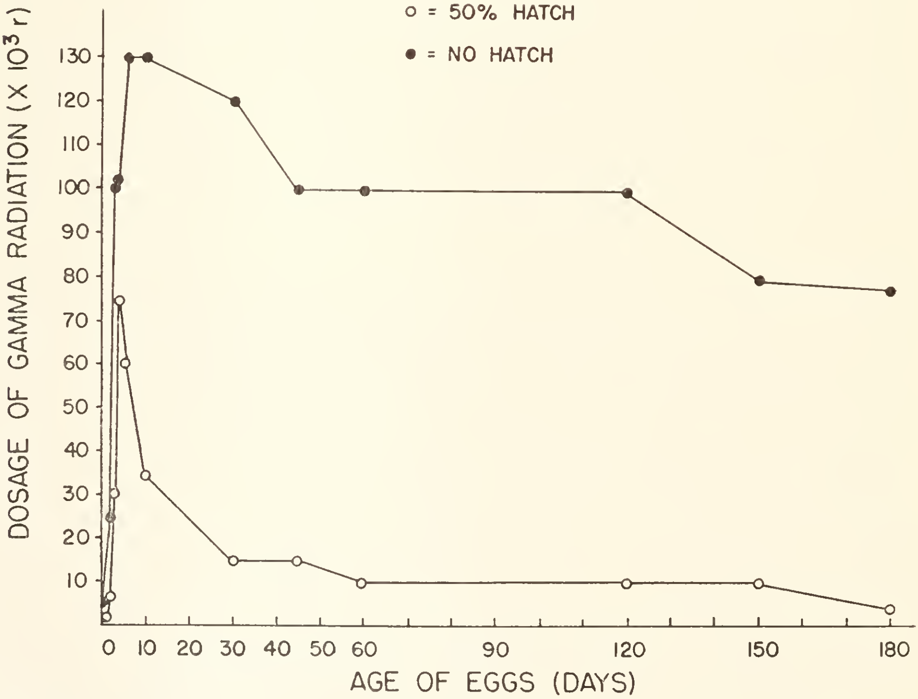

FIGURE 1. The relation between the radiation dosage required to inhibit hatching and the age of the eggs of *Aedes aegypti*.

the viability of the F-1 progeny. Attempts to rear fertile progeny from these irradiated eggs produced results almost identical to the results obtained with 2-day-old and 16-day-old eggs except that in this case, after numerous attempts, a few adults were finally obtained from eggs exposed to 2500 r, which did mate and lay fertile eggs. Thus, again, although the dosage required to impair the impetus to hatch varied with the age of the egg, the dosage required to destroy the viability of the egg was constant regardless of age. It is of interest to note, again, that the dosage was directly related to the length of time larvae developed or survived following exposure to radiation, in that the higher the dosage the quicker larval development was arrested and the larvae died.

Summary

1. It has been shown that when normal *A. aegypti* females are mated and given a blood meal 24 hours after exposure of the males to gamma radiation in doses up to 30,000 r, egg production is not significantly affected. If, however, mating is delayed 8 or 15 days following irradiation of the males, egg production decreases and the period of oviposition increases apparently because fewer males are able to copulate even though they still contain motile spermatozoa.

2. Although eggs continue to be produced in some quantity whether mating is immediate or delayed, fewer larvae hatch from eggs produced by females mated to males exposed to 2500 r, while a very few larvae hatch from eggs produced from matings in which the males received 10,000 r. However, it was possible to grow larvae successfully to fertile adults capable of mating and producing viable eggs only from matings in which the males had received a maximum of 7500 r.

3. It has been shown, too, that the egg production of female mosquitoes, exposed first to gamma radiation and mated 24 hours later to normal males, is significantly reduced among those receiving 2500 r, and almost entirely eliminated among those exposed to 10,000 r. However, larvae which could be grown to fertile adults were obtained only from eggs produced by females exposed to a maximum of 5000 r. When mating was delayed, no eggs were produced by females exposed to doses in excess of 3500 r, although viable larvae hatched from eggs of females exposed to 3500 r.

4. Females inseminated prior to being exposed to radiation produced approximately the same number of eggs at the various dosage levels as females inseminated subsequent to exposure. However, significantly fewer larvae hatched from these eggs than from the eggs laid by females inseminated subsequent to exposure.

5. To determine the effects of radiation during the cycle of egg development which occurs in *A. aegypti* following a blood meal, inseminated females were exposed to gamma radiation at various intervals following engorgement. It was found that egg production was almost entirely inhibited in females exposed to 10,000 r 4 hours after the blood meal, whereas it required in excess of 100,000 r to inhibit egg production in females in which exposure had been delayed 42 hours after the blood meal. On the other hand, although it required higher and higher doses of radiation to inhibit egg production the longer irradiation was delayed, nevertheless, the eggs became more and more sensitive to radiation as they matured within the body. Thus, whereas viable larvae resulting in fertile adults developed from eggs produced by females exposed to 5000 r 4 or 24 hours after the blood

meal, viable larvae could be obtained from the eggs of females exposed to only 3500 r when exposure was delayed 42 hours.

6. Finally, it has been shown that although the dosage required to inhibit hatching of the eggs of *A. aegypti* exposed to gamma radiation varied enormously according to the age of the egg, nevertheless, eggs exposed to doses in excess of 2000 r, regardless of age, could not be grown to adults. Again, however, as in the experiments in which either males or females were exposed to radiation, whenever larvae could be grown successfully to adults, the resulting adults proved to be fertile and capable of producing viable eggs if they were physically capable of mating.

QUATERNARY AMMONIUM BASES IN THE COELENTERATES[1]

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The stinging organelles or nematocysts of coelenterates appear to serve two functions. By injecting an irritating substance they serve as effective weapons of defense, while accompanying paralyzing action, probably by a different agent or combination of agents, is useful in quieting prey in the process of feeding. In spite of a considerable amount of work over the past fifty years, the chemical nature of the nematocyst toxin is still unknown. Some of the earlier literature has been summarized elsewhere along with an account of some recent work (Welsh, 1956).

Aqueous extracts of nematocyst-bearing tentacles of representatives of each of the three classes of coelenterates, when injected in crabs, produce a preliminary excitation. Spontaneous autotomy of legs may accompany the excitation. After a time the crabs become paralyzed and, if the dose is sufficient, they fail to recover. An injection of tetramethylammonium chloride mimics the paralyzing action of extracts, while an earlier or simultaneous injection of a salt of tetraethylammonium antagonizes the paralyzing actions both of extracts and a tetramethylammonium halide (Welsh, 1956). Since Ackermann, Holtz and Reinwein (1923) had isolated tetramethylammonium hydroxide ("tetramine") from sea anemones, the possibility existed that this substance, or some derivative, was the active paralyzing principle.

Largely through the efforts of Ackermann and co-workers several other quaternary ammonium bases have been isolated from sea anemones and chemically identified. Ackermann, Holtz and Reinwein (1924a) isolated and identified N-methylpyridinium hydroxide from *Actinia equina*, along with a compound tentatively named "actinin." Later, the same authors (1924b) presented evidence that led them to suggest that actinin was probably the alkaloid stachydrine. Ackermann (1927), however, determined actinin to be γ-butyrobetaine, and not stachydrine. Recently Ackermann (1953) found homarine and trigonelline in extracts of the sea anemone, *Anemonia sulcata,* along with an unidentified base which he first named "anemonin," but later (1954) changed to "zoo-anemonin." Evidence for the occurrence of trigonelline in the siphonophore, *Velella spirans,* had been presented earlier by Haurowitz and Waelsch (1926). Zoo-anemonin was identified as the dimethylbetaine of imidazole acetic acid by Ackermann and Janka (1953), but the correctness of the structural formula that they gave will be discussed later.

The present study began as an attempt to determine whether or not tetramine was generally present in coelenterates. Since paper chromatography was used, followed by reagents that help in the visualization of quaternary ammonium bases, it soon became obvious that several such compounds were present. The work was extended in an attempt to identify these. Unfortunately certain of the papers of Ackermann and others were not known to us until we had spent considerable time in the identification of those bases. We have now identified, with reasonable certainty, tetramethylammonium (I), homarine (III), trigonelline (IV) and γ-butyrobetaine (V) in representatives of all three classes of coelenterates (see Fig. 1). We find what probably corresponds with Ackermann's zoo-anemonin (VI) in a horny coral and in two species of sea anemone. With the method used, we have been unable to identify N-methylpyridinium (II) in any coelenterate, although using the same method we can demonstrate its presence in certain molluscan tissues. Some spots which react as quaternary ammonium bases have not been identified.

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[1] This investigation was supported in part by research grant B-623 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

Preliminary tests of the toxicity of the identified bases have been made.

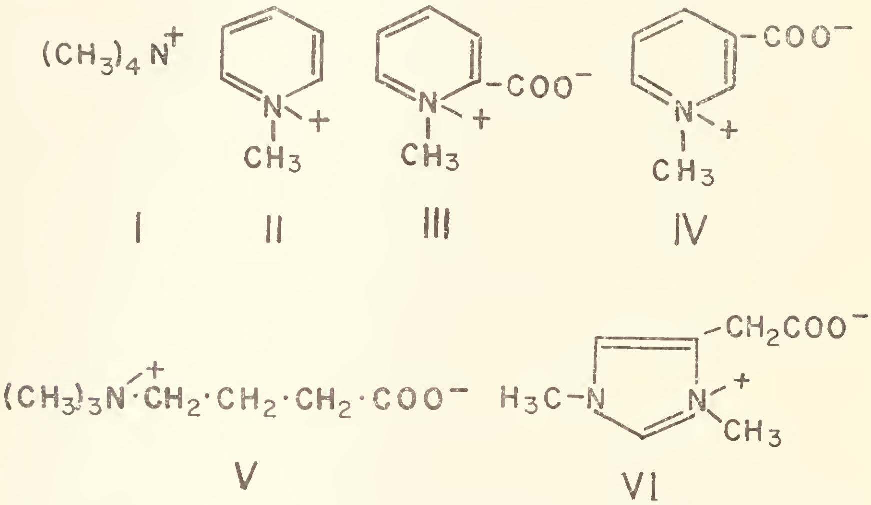

FIGURE 1. Structural formulae of compounds included in this study. I = tetramethylammonium; II = N-methylpyridinium; III = homarine; IV = trigonelline; V = γ-butyrobetaine; VI = zoo-anemonin.

MATERIALS AND METHODS

Extracts were made from the following:

Class Hydrozoa

Hydra littoralis—some supplied by Dr. W. F. Loomis; some mass cultured according to Loomis and Lenhoff (1956); others collected locally.

Physalia physalis L.—the Portuguese man-of-war, fishing filaments only; collected in Bermuda and Bimini, B. W. I.

Class Scyphozoa

Cyanea capillata (L.)—brown or red jellyfish, tentacles only; collected in Puget Sound, Washington.

Class Anthozoa

Plexaura flexuosa—a horny coral, whole animal; collected in Bermuda.

Metridium dianthus (Ellis)—sea anemone, whole animal or tentacles; collected at Nahant and Rockport, Massachusetts.

Condylactis gigantea (Weiland)—pink-tipped sea anemone, tentacles only; collected in Bermuda.

Whole animals were macerated in a Waring Blendor and 4–5 volumes of acetone added. Tentacles were cut off and placed in 4–5 volumes of acetone. The tissues in acetone were stored in a refrigerator. When needed, a given volume (20 or 25 ml.) of the acetone extract was decanted, filtered and the acetone removed under reduced pressure. The remaining material was dried and washed with about 10 ml. of petroleum ether, the bases were then taken up in 1 or 2 ml. of 95% ethanol for chromatography. No attempt was made to secure quantitative yields but the results give a good idea of the relative amounts of different bases that were extracted from a given species.

Extracts and knowns were chromatographed using wide strips of Whatman No. 3 MM filter paper. After trying a variety of acidic and basic solvent systems we found that the most satisfactory separation was obtained with a mixture of 95 parts of 95% ethanol and 5 parts of ammonium hydroxide (28%), as recommended by Bregoff, Roberts and Delwiche (1953). When two-dimensional chromatograms were run, the second solvent system was n-butanol-acetic acid-water (10:3:8–9). The jars were allowed to saturate for at least seven hours and the papers equilibrated 2–3 hours. The ascending method was used. Jars were kept in a chamber in which the temperature was maintained at 25° C. ± 1°. The most satisfactory chromatograms were obtained after runs of 9–10 hours. After drying, the chromatograms were examined under ultraviolet light (short-wave "Mineralight") and any ultraviolet absorbing areas outlined with pencil. Of the several reagents used to visualize the areas occupied by quaternary ammonium compounds, the most generally satisfactory was Dragendorff's solution ($KBiI_4$ reagent) as modified and used by Bregoff, Roberts and Delwiche (1953).

To identify zoo-anemonin, a solvent system consisting of n-butanol-dioxane-water in the proportions of 4:1:5, was also used to permit comparison of the Rf value with that obtained by Ackermann and Janka (1953).

In order to make more certain the identification of homarine and trigonelline, both of which absorb strongly in the ultraviolet, absorption spectra of eluates were compared with those of synthetic compounds using the Cary recording spectrophotometer. Rather large amounts of extracts were placed on paper and run with ethanol-ammonia solvent. Ultraviolet absorbing areas were outlined and a strip was cut from one side for development with $KBiI_4$. The desired areas were cut out and eluted with distilled water. They were appropriately diluted and absorption spectra were obtained. We are greatly indebted to Mr. and Mrs. Paul Brown for their cooperation in this part of the study.

The toxicities of tetramethylammonium bromide, N-methylpyridinium, homarine, trigonelline, γ-butyrobetaine and N,N′-dimethylimidazole acetic acid were determined on the fiddler crab, *Uca pugilator*, from Florida. Each was tested on one or more lots of 5 crabs, by injecting 0.02 or 0.05 ml. of a 1% solution at the base of one of the walking legs.

The known quaternary ammonium standards used in this study were from the following sources: tetramethylammonium bromide, Eastman Organic Chemicals; N-methylpyridinium bromide, kindness of Dr. J. A. Aeschlimann, Hoffmann-La Roche Inc.; homarine, kindness of Dr. E. L. Gasteiger; trigonelline, General Biochemicals Inc.; while γ-butyrobetaine was prepared from γ-carbomethoxypropyltrimethylammonium bromide (generously supplied by Dr. R. W. Fleming, Parke Davis and Co.) after the method suggested by Bregoff, Roberts and Delwiche (1953). A sample of the dimethylbetaine of imidazole acetic acid, as the hydrochloride ($C_7H_{10}O_2N_2 \cdot HCL \cdot H_2O$), was kindly furnished by Dr. D. Ackermann. A second sample was made from imidazole acetic acid (supplied by Dr. H. Bauer, National Institutes of Health) in the laboratory of Dr. R. B. Woodward. The two samples had similar melting points and similar Rf values. Dr. Woodward informs us that the structural formula for anemonin (zoo-anemonin) as given by Ackermann and Janka (1953) is in error and that the correct formula is as given in the series of structural formulae. The more descriptive name for this substance would, therefore, be N,N′-dimethylbetaine of imidazole acetic acid.

RESULTS

Chromatograms

Extracts of tentacles of whole animals of the six selected species, representing each of the three classes of coelenterates, were chromatographed according to the procedure outlined in the section on Methods. Each extract was run many times along with one or more samples of known quaternary ammonium bases. The relative Rf values of these bases are given at the left of Figure 2. All results are for the ethanol-ammonia solvent system. It may be seen that tetramethylammonium bromide (I) gave an Rf value of 0.75; N-methylpyridinium bromide (II) an Rf of 0.64; homarine HCl (III) an Rf of 0.54; trigonelline (IV) an Rf of 0.32; and γ-butyrobetaine bromide (V) an Rf of 0.27. For each species, the compounds found and identified with reasonable certainty, with the exception of N,N′-dimethylbetaine of imidazole acetic acid (VI), are represented by shaded areas.

Tetramine was present in each of the species examined, being the only base found in *Hydra*. The two sea anemones yielded smaller amounts than the other species and in *Metridium* this spot was most distinct when an extract of tentacles, rather than of whole animal, was used. Extracts of the gorgonian, *Plexaura flexuosa,* contained relatively large amounts of tetramine, as suggested by the larger shaded area. It is of interest to note that separate extracts were made of purple and brown varieties of colonies of *Plexaura*. The chromatograms of these extracts were so similar that they are represented by the one set of spots of the four bases that were identified.

In none of the species examined did we find an indication of the presence of N-methylpyridinium. Since the methods employed have enabled us to identify this substance in extracts of certain molluscan tissues, we believe it to be absent, or present in very small amounts, in the coelenterates investigated. Homarine is a compound now known to be widely distributed among marine invertebrates (Gasteiger, Gergen and Haake, 1955). We found it in all five marine species of coelenterates examined. Although present in relatively large amount in our extracts of

Metridium, it was determined with least certainty in the pink-tipped sea anemone, *Condylactis.*

In the ethanol-ammonia solvent, the Rf values of trigonelline and γ-butyrobetaine were so similar that the spots overlapped. In the case of *Plexaura* extracts, where a relatively large amount of trigonelline was present, two-dimensional chromatograms were run. This permitted a clear-cut separation of trigonelline and γ-butyrobetaine. Trigonelline was not found in our extracts of *Physalia,* although it was identified with reasonable certainty in the other four species. Extracts of the pink-tipped sea anemone, *Condylactis,* contained large amounts of γ-butyrobetaine, while extracts of *Physalia* and *Metridium* appeared to lack this substance.

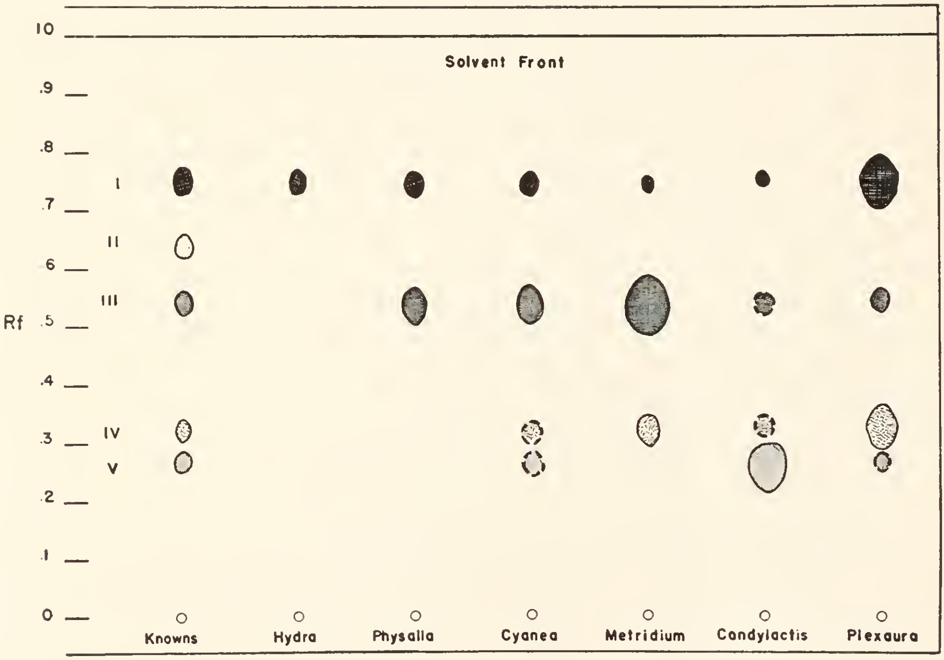

FIGURE 2. Composite of chromatograms giving Rf values for five of the compounds with which this study was concerned. In instances where identification was tentative, the spots are shown with broken boundary lines. I = tetramethylammonium; II = N-methylpyridinium; III = homarine; IV = trigonelline; V = γ-butyrobetaine. Solvent = ethanol-ammonia.

During the period when most of the work reported here was in progress we were not aware of the identification of zoo-anemonin as the dimethylbetaine of imidazole acetic acid. Now having samples of the synthesized material we find what we believe to be zoo-anemonin in *Metridium, Condylactis* and *Plexaura.* Unfortunately, when ethanol-ammonia is used as a solvent system, the Rf value of zoo-anemonin is between 0.2 and 0.3. This is so similar to that for γ-butyrobetaine that some other solvent system must be used for their separation. We have tried n-butanol-dioxane-water (4:1:5) as used by Ackermann and Janka (1953) for zoo-anemonin. With this they obtained an Rf of 0.17. Extracts of *Metridium* and

Plexaura run with this solvent give a relatively large spot appearing between Rf 0.1 and 0.2 and probably representing zoo-anemonin.

Identification by ultraviolet absorption

Spots of N-methylpyridinium, trigonelline and homarine are readily detected as absorbing areas when dried, untreated chromatograms are examined with short-

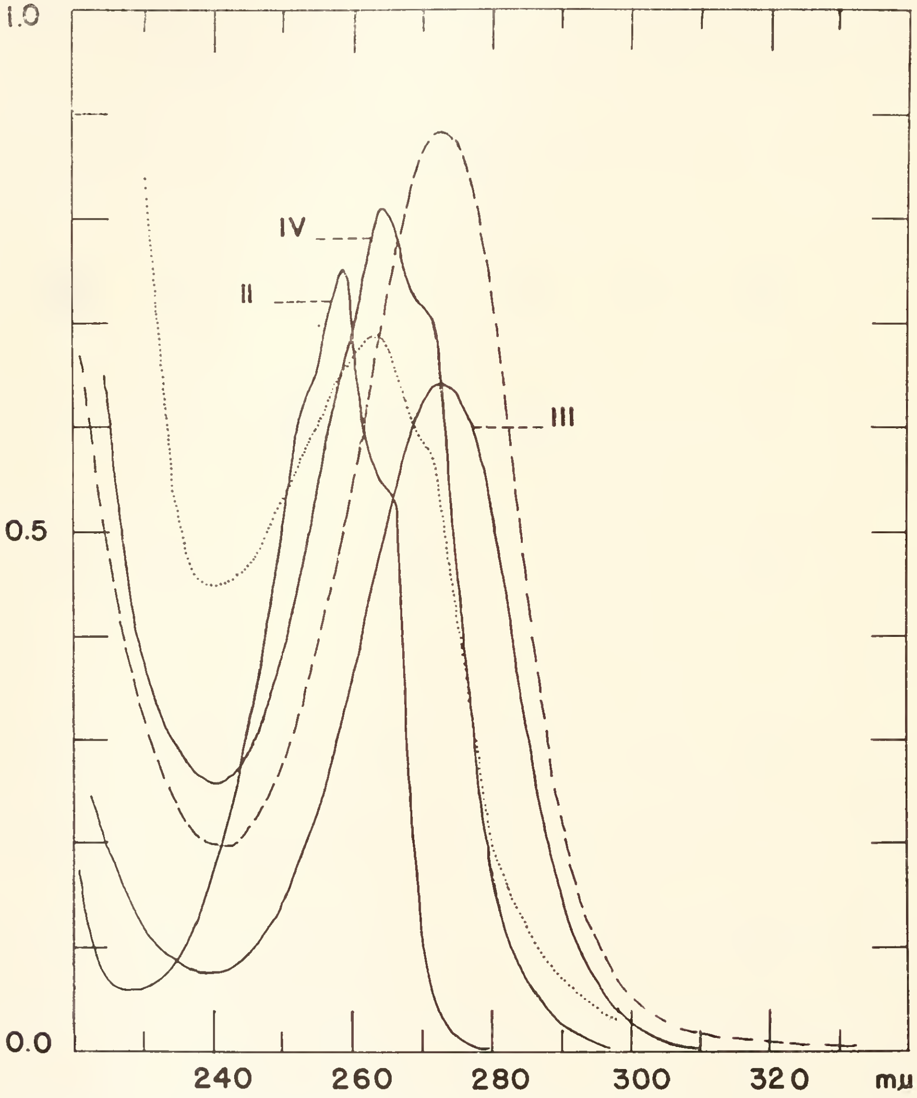

FIGURE 3. Ultraviolet absorption curves for N-methylpyridinium (II), homarine (III), trigonelline (IV), and for eluates of spots from chromatograms of *Metridium* tentacle extracts believed to represent homarine (dashed line) and trigonelline (dotted line). Ordinate = arbitrary units of absorption.

wave ultraviolet light. This was very helpful in the location of regions on the paper where these compounds occurred. Thus, an absorbing area was never found on chromatograms of coelenterate extracts in a region where N-methylpyridinium should have occurred, if it had been present. It should be noted, however, that N-methylpyridinium bromide, when run in ethanol-ammonia, gives two spots, the lower of which is close to homarine. This could give rise to some confusion but we believe, in this instance, that it has not done so.

The ultraviolet-absorbing characteristic of these pyridine derivatives was further used in the identification of homarine and trigonelline. Samples of crystalline N-methylpyridinium, trigonelline and homarine were run in a Cary recording spectrophotometer. Tracings of their absorption curves are combined in Figure 3. Since we were interested mainly in the wave-length at which maximum absorption occurred, their extinction coefficients were not determined. N-methylpyridinium absorbed maximally at 258 mμ, trigonelline at 264 mμ, and homarine at 272–3 mμ.

Extracts of *Metridium* and *Physalia* were streaked on 5-inch-wide strips of paper. After running in ethanol-ammonia the papers were dried and the areas believed to be occupied by trigonelline or homarine were outlined under an ultraviolet light source. These areas were then cut out and eluted with distilled water. After appropriate dilution (determined by trial) the absorption curves for these extracts were also obtained. Figure 3 shows curves for eluates of the areas of *Metridium* chromatograms believed to represent homarine and trigonelline. Maximum absorption of the eluate supposed to contain trigonelline is seen to correspond precisely with that of the authentic samples of trigonelline (IV). The absorption curves for both samples have similar characteristic shoulders.

The absorption curve of a *Metridium* eluate, believed to contain homarine, is identical in shape with that for synthetic homarine. In a similar manner, homarine was identified in extracts of tentacles of *Cyanea* and *Physalia*. The procedure was not used with the other species.

On chromatograms of tentacle extracts of *Metridium* and *Physalia,* run in ethanol-ammonia, an ultraviolet-absorbing area was found with an Rf of about 0.2. Eluates of this area gave absorption curves with a maximum absorption at 248 mμ. The substance responsible for this was not identified.

The action of quaternary ammonium bases on fiddler crabs

Aqueous extracts or homogenates of tentacles of certain coelenterates have been shown to influence the autotomy reflex of crustaceans (Welsh, 1956). Likewise, certain quaternary ammonium bases were found to facilitate or to reduce the tendency to autotomize legs. In order to determine whether or not the bases under investigation in the present study would reproduce the actions of coelenterate extracts, the following experiments were performed. A given volume of *Metridium* tentacles was homogenized with an equal volume of sea water. After centrifuging, 0.05 ml. of the clear supernatant was injected into each of five *Uca pugilator.* In three minutes, six legs had spontaneously autotomized and the crabs were showing signs of severe paralysis, from which none recovered. The original extract was diluted 1:10 with sea water and five crabs injected with 0.05 ml. each. In five minutes, four of the crabs had dropped 25 legs (one crab failed to autotomize any legs). In a few more minutes, they were completely paralyzed and none recovered.

A dilution of 1:100 with sea water produced 12 autotomies in 15 minutes. After 24 hours two crabs were dead and after 48 hours two additional crabs. Thus, an aqueous extract of *Metridium* tentacles contains factors which, in considerable dilution, produce spontaneous autotomy of legs followed by paralysis and death, in this species of crustacean.

An abundance of *Hydra littoralis,* from mass cultures, made it convenient to test the action of a hydra extract on a crustacean. Approximately 2000 hydra, unfed for one week, were blotted and weighed. The wet weight was 410 mg. They were homogenized and one ml. of distilled water added. After centrifuging, 0.02 ml. of the clear supernatant was injected into *Uca pugilator.* The following is a typical record:

1:30 PM Injected 0.02 ml. at base of second left walking leg of a specimen of *Uca* weighing 3.16 gm.
1:31 PM First and second left legs "paralyzed"
1:33 PM Crab cannot right itself when turned on back
1:34 PM Only slight limb movements
1:40 PM All spontaneous movements have ceased and no response to stimulation
2:45 PM No indication of recovery; appears dead, but on removing carapace the heart is found beating.

This extract when diluted 1:10 with sea water was almost as effective in causing paralysis of *Uca* as the undiluted extract. However, when heated at 100° C. for five minutes an injection of 0.02 ml. was entirely without effect on *Uca.*

Next, a series of tests was made to determine whether or not any one of the six bases used in this study, and available in crystalline form, would mimic in any respect the extract of *Metridium* tentacles. Each base was made up as a one per cent solution in sea water and 0.05 ml. injected into each of five *Uca pugilator.* Of the six bases only tetramethylammonium bromide appeared to have significant action. This substance produced a type of paralysis from which only three of five crabs recovered.

It seemed possible that a mixture of the bases in question might have an action that individual members lacked. Therefore, they were combined and injected. The action on the crabs was unspectacular and did not differ from that produced by an equivalent amount of a tetramethylammonium salt. From these injection experiments it would appear that the toxic action of an aqueous extract of *Metridium* tentacles, or whole hydra, on the fiddler crab, *Uca pugilator,* could not be due solely to the presence of the quaternary ammonium bases with which this study was chiefly concerned.

The presence of a tetramethylammonium compound in all species of coelenterates that were examined; its occurrence as the only quaternary base identifiable in *Hydra littoralis* (by the methods used) and its known effects on crustaceans (Welsh, 1956) would all appear to support the earlier suggestion of Ackermann, Holtz and Reinwein (1923) that tetramethylammonium hydroxide (tetramine) might be the paralyzing factor in nematocyst toxin. Two observations made in the present study make this suggestion unlikely. They are (1) that a dose of hydra extract calculated to contain the active material from 0.14 mg. of dry hydra is fatal to a specimen of *Uca*, while 0.5 mg. of crystalline tetramethylammonium bromide is not, and (2) that heating for 5 minutes at 100° C. destroys or greatly lowers the activity of an aqueous hydra extract. This should have little, if any, effect on a tetramethylammonium salt.

DISCUSSION

Studies made on extracts of whole coelenterates, their tentacles, or their acontia, will not conclusively identify the chemical constituents of nematocyst contents and, therefore, coelenterate or nematocyst toxins, as was recently pointed out by Phillips and Abbott (1957). Such studies may, however, give valuable clues to the nature of the toxic substance, and toxic components of extracts of tissues, rich in nematocysts, may then be sought in extracts of the isolated and cleaned stinging organelles. Methods for isolating undischarged nematocysts have been developed (Phillips, 1956; Phillips and Abbott, 1957) and are being adopted by others (*e.g.* Dodge and Lane, 1958; Lane and Dodge, 1958).

The work reported here was an attempt to learn more about the distribution of tetramine and other quaternary ammonium bases in representative coelenterates. While several bases were found in marine coelenterates, only tetramine was present in the fresh water hydra in sufficient amounts to be identified with the methods employed. This finding, and the observation that tetramine was the only base employed in this study that had significant paralyzing action on *Uca*, provide further evidence that this substance may be a constituent of nematocyst toxin. Almost certainly it is not solely responsible for the paralyzing effects of coelenterate stings. One or more proteins could be additional components. This is suggested by the decreased activity of hydra extracts that have been heated (see above) and by the loss of toxicity by isolated nematocysts that have been treated with ether, alcohol or drying (Phillips and Abbott, 1957). Against the view that proteins may be important in nematocyst toxin is an earlier observation that deproteinization with trichloracetic acid did not significantly alter the toxicity of extracts of acontia of *Adamsia palliata*, when *Carcinus* and *Astacus* were used as test animals (Cantacuzène and Damboviceanu, 1934a). Further observations on the trichloracetic acid extract of *Adamsia* acontia suggest that the crustacean-paralyzing factor is a relatively small and quite stable molecule (Cantacuzène and Damboviceanu, 1934b).

It is not unreasonable to theorize that an association of tetramine with a protein might produce a substance more toxic than tetramine alone. This is based partly on the evidence that two alkylated tetracovalent nitrogens, properly spaced in a molecule, can produce highly active junctional blocking agents such as curare and the many synthetic, curariform, bis-quaternary substances. Protein denaturation by heat, or otherwise, might alter the spacing of the tetramines on the protein or set them free and thereby reduce, but not abolish, the paralyzing action of an extract. In support of such a suggestion is the observation that nematocysts have a high affinity for methylene blue, a basic dye with two methylated nitrogens which, through resonance, may become tetracovalent. This implies that there are molecules within the nematocyst (presumably protein) that bind methylene blue and that might bind other quaternary ammonium bases.

Although certain pyridinium derivatives have a weak curariform action in vertebrates (Craig, 1948) those studied here, as well as the other betaines, were characterized by their lack of paralyzing action on *Uca*. Since homarine, one of the compounds in question, occurs widely in marine invertebrates but not in those from fresh water it has been suggested that it may serve an osmoregulatory function (Gasteiger *et al.*, 1955). This may be the role of some of the other nitrogenous bases of marine invertebrates.

When this study was first begun we tentatively identified one of the bases of *Metridium* and *Physalia* extracts as urocanylcholine (Welsh, 1956). This identification was based partly on the ultraviolet absorption of eluates of chromatograms and their comparison with known urocanylcholine. Although the curves and peaks of absorption correspond rather precisely at a certain pH value, we later learned that the absorption maximum of the eluted material did not change with pH as does that of urocanylcholine (Erspamer and Benati, 1953). Later we learned that the suspected urocanylcholine was actually homarine.

Although we do not yet know what is responsible for the paralysis produced by the nematocysts of coelenterates, the renewed interest in this question should eventually provide an answer.

SUMMARY

This was a study of the identification and distribution of quaternary ammonium bases in representative coelenterates. The purpose was to determine if bases were present with paralyzing actions greater than that of tetramethylammonium (tetramine) which was found to occur in all species examined. Four other bases (homarine, trigonelline, γ-butyrobetaine and the dimethylbetaine of imidazole acetic acid) were found in some species. The bases other than tetramine were found to have no observable paralyzing action on *Uca pugilator*, in the doses employed. However, it is not possible to account for the powerful paralyzing actions of cold, aqueous extracts of *Metridium* tentacles or whole hydra on the basis of their tetramine content. It is suggested that this base, in conjunction with a specific protein, might be responsible for the paralyzing action of nematocysts.

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Volume 115 Number 1

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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SEP 5 - 1958
WOODS HOLE, MASS.

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AUGUST, 1958

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

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THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. All subscriptions expire with the December issue and are renewable prior to the next succeeding February issue. Single numbers, $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 1 and September 1, and to Dr. Donald P. Costello, P. O. Box 429, Chapel Hill, North Carolina, during the remainder of the year.

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